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BLOOD PLASMA PROTEIN PRODUCTION AS INFLUENCED BY VARIOUS DEGREES OF HYPOPROTEINEMIA AND BY AMINO ACIDS*

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Blood plasma proteins are an *active* part of the complex integrated system of body proteins This thesis has been supported by various contributions coming from this laboratory There is ample evidence that a constant ebb and flow exists between plasma and cell proteins, a *dynamic equilibrium* We believe that this protein exchange may be at least as important in cell nutrition as the commonly accepted passage of amino acids from the digestive tract to the special cell proteins (2, 10, 14, 7)

This protein exchange, this ebb and flow between plasma and cell proteins is in large part dependent upon the *reserve store* of protein *within* various body cells (*e g*, in liver and in muscle cells) But this reservoir of protein material is in no sense an idle pool of protein It participates in the constant steady state maintained between the plasma proteins on the one hand and the integral cell proteins on the other It may be likened to a beaver pond beside a rapid stream, which rises and falls as does the stream and is further modified by drainage from and seepage into the adjacent banks

As this reserve protein must be largely or wholly within the cell boundaries it is probably specific *cell protein*, yet in some way different from integral cell proteins because it can be withdrawn or supplemented on occasion Our knowledge of cell proteins does not permit us to specify differences between these "reserve," "intermediate" labile cell proteins and the integral, relatively fixed cell proteins In other papers (2, 9) we have used various terms to cover this labile protein material which flows in and out of body cells "intermediates," or "large aggregates approximating proteins but not fixed tissue proteins" Perhaps the term "intermediate protein" may appear more suitable until the specifications can be more clearly written

* We are indebted to Eli Lilly and Company for aid in this work

This concept is in harmony with observations reported by Schoenheimer and Rittenberg (11) using a totally different approach

The *production of new plasma protein* which can be removed from the body (by plasmapheresis) is controlled in a measure by the concentration of plasma protein in the circulation Table 1 indicates that as the concentration of circulating plasma protein rises the amount of new plasma protein which can be removed falls rapidly We believe that some (and

TABLE 1

Rate of Blood Plasma Protein Regeneration

Decreases When a Low Plasma Protein Level Rises toward Normal

Dog 36-196

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Blood plasma Average concentration		R B C hema tocrit average	Plasma volume
				Total protein	A/G ratio		
		gm	*gm*	*per cent*		*per cent*	*cc*
	Kennel			5 81	1 30	45 0	
1	Fasting	0	35 5	5 28	0 93	51 5	491
2	Low protein	15	16 6	4 07	0 76	56 6	403
3	Liver basal	85	7 6	4 34	0 67	50 4	504
4	Liver basal	85	15 2	4 58	0 65	47 5	521
5	Liver basal	85	9 6	4 65	0 70	48 9	487
6	Liver basal	85	5 2	4 60	0 82	48 0	474
7	Liver basal	85	6 9	4 49	0 93	47 2	489
8	Liver basal	85	1 1	4 73	—	44 8	513
9	Liver basal	85	23 0	4 44	0 77	46 9	479
10	Liver basal	85	17 1	3 98	0 67	49 8	489
11	Liver basal	85	16 2	4 03	0 67	48 5	462
12	Liver basal	85	15 9	4 00	0 61	49 2	—

possibly all) of this apparent reduction in the formation of new plasma protein as the concentration rises may be accounted for by escape of plasma protein into the body cells

The present study continues our research program relative to the factors controlling the production of new plasma protein A normal adult dog is depleted by plasmapheresis (bleeding with return of red blood cells suspended in a saline solution) and is maintained hypoproteinemic by suitable plasmapheresis and a diet limited in its protein content It is evident, in Tables 1 and 1-*a*, that much less plasma protein can be removed in maintaining a steady high hypoproteinemic level than in carrying a steady low hypoproteinemic level If the rate of regeneration of plasma protein is the same at 4 6 per cent as at 4 00 per cent the rate of *disappearance*

of the new plasma protein into the body cells must be faster at the higher level We have no measure of the true rate of regeneration of new plasma protein, we measure only what we can remove from the blood stream while maintaining comparable conditions We realize that plasma protein can rapidly disappear from the blood stream but concerning the normal rate of this disappearance we have no measurement This interesting problem has been discussed in a recent review (7)

TABLE 1-*a*

Weight and Nitrogen Balance

Dog 36-196

Period 7 days	Diet	Weight	Nitrogen balance					
			Intake		Output			Intake minus output
			in diet	In excess R.B C injected	in plasma	in urine	in feces	
		kg	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
	Kennel	13 6						
1	Fasting	12 1	0	+3 9	5 8	15 6	*	−17 5
2	Protein low	12 1	2 8	−3 7	2 7	10 7	*	−14 3
3	Liver basal	11 8	14 0	+0 1	1 3	10 5	5 1	−2 8
4	Liver basal	11 9	14 0	+1 2	2 6	9 9	2 8	−0 1
5	Liver basal	12 0	14 0	+0 2	1 6	9 4	3 2	0 0
6	Liver basal	12 0	13 8	−2 5	0 8	9 2	2 3	−1 0
7	Liver basal	12 1	13 8	+2 7	1 1	9 2	3 0	+3 2
8	Liver basal	12 1	13 9	−1 0	0 2	10 3	3 6	−1 2
9	Liver basal	12 1	13 9	+4 1	3 8	9 0	2 3	+2 9
10	Liver basal	12 3	13 9	+2 3	2 8	10 0	3 1	+0 3
11	Liver basal	12 3	13 9	+1 5	2 7	10 4	3 2	−0 9
12	Liver basal	12 4	13 9	+1 2	2 6	9 8	3 2	−0 5
Totals			141 9	10 0	28 0	124 0	31 8	−31 9

* *Included in following period*

It is obvious that in such experiments with plasmapheresis it is necessary to maintain the plasma protein concentration at a uniform level if we would test accurately the effect of diet factors on plasma protein production—Table 2

Methods

Previous papers in this series (5) have outlined the procedure employed in this present work. Attention should be called again to the figures given for blood plasma protein concentration They are obtained by Kjeldahl nitrogen determinations on the plasma of the pooled daily bleeding collected in saturated sodium citrate solution 1 ml per 100 ml blood They are lower than the levels of the plasma protein actually circulating in the dog immediately prior to such bleedings for reasons noted in an earlier report (8)

They may be 10 to 15 per cent lower than these circulating levels but are within one per cent of the actual concentration of protein in the plasma removed

The gelatin fed is of the same lot as that used previously (4) The naturally occurring forms of the amino acids were used except for racemic mixtures of isoleucine and methionine

The amount of plasma protein removed on any day depends upon the concentration of protein in the plasma and the amount of plasma in the blood removed on that day The amount of blood removed is adjusted to two factors the amount the dog will tolerate without clinical disturbance, such as loss of appetite, and, second, the amount the workers estimate must be removed to achieve a certain level on a given régime When the level is high and the aim is to reduce the protein mass rapidly, 25 per cent of the blood volume is a convenient and safe quantity to remove each day When a significant hypoproteine-

TABLE 2

Blood Plasma Protein Regeneration

Favored When Certain Amino Acids Supplement Gelatin

Dog 36-196

Period		Diet	Protein intake per 7 day period	Plasma protein removed per 7 day period	Blood plasma Average concentration		R.B C hematocrit, average
					Total protein	A/G ratio	
No	*days*		*gm*	*gm*	*per cent*		*per cent*
12	7	Liver basal	85	15 9	4 00	0 61	49 2
13	4	Low protein	15	8 9	3 80	0 62	51 0
14	2	Low protein + gelatin + amino acids A	134	2 0	3 97	0 54	49 0
15	7	Low protein + gelatin + amino acids A	134	18 6	4 27	0 79	50 2
16	4	Low protein	15	8 7	4 02	0 79	52 0
17	3	Low protein + gelatin + amino acids B	127	11 0	4 13	0 72	48 9
18	7	Liver basal	85	16 5	4 20	0 78	50 2

mia is reached, 3 5 to 4 0 per cent, the size of subsequent bleedings may be based on experience We have tried to maintain the concentration each day at the same constant hypoproteinemic level during weeks and months of testing of factors which would have varied this steady level were not the size of the daily bleedings properly adjusted The effect of a test factor has been related to the quantity of plasma protein which had to be removed in order to maintain this level We believe this measurement to be valid only if the beginning and ending concentrations of plasma protein are reasonably close and have each been maintained reasonably close by equivalent bleedings over a sufficiently long period of time In other words a steady state must have been demonstrated to have preceded and followed any measurement of quantitative significance Such qualifications, for example, are fulfilled in the experiments of Tables 2 and 2-*a*

In past experiments we have not recognized any significant change in the quantity of plasma required to be removed in maintaining a constant level at any point between 3 7 and 4 3 gm per cent Below this range our past

data are inconclusive since clinical disturbances are frequent and stop the experiment Above this range, the experiments in Tables 1 and 1 *a* indicate that the quantity to be removed decreases sharply

EXPERIMENTAL OBSERVATIONS

The data presented in Tables 1 and 1 *a*, 2 and 2 *a*, are all obtained from one continuous 16 week experiment in a dog, 36 196, additionally valuable because of the similar experiments previously performed with its excellent cooperation (6, 4) In Tables 1 and 1 *a* the data are given as the totals or averages of those obtained on the individual days of the consecutive

TABLE 2-*a*

Weight and Nitrogen Balance

Dog 36-196

Period		Diet	Weight	Nitrogen balance per 7 day period					
				Intake		Output			Intake minus output
				in diet	in excess R.D.C injected	in plasma	in urine	in feces	
No	days		kg	gm	gm	gm	gm	gm	gm
12	7	Liver basal	12 4	13 9	+1 2	2 6	9 8	3 2	−0 5
13	4	Low protein	—	2 7	+0 2	1 5	12 1	3 0	−13 7
14	2	Low protein + gelatin + amino acids A	12 2	27 5	−2 4	0 3	16 6	3 8	+4 4
15	7	Low protein + gelatin + amino acids A	12 2	27 5	+2 9	3 1	17 5	3 8	+6 0
16	4	Low protein	12 3	2 7	+1 1	1 4	10 6	3 0	−11 2
17	3	Low protein + gelatin + amino acids B	12 4	24 6	0 0	1 9	13 1	3 7	+5 9
18	7	Liver basal	—	13 9	+2 5	2 7	9 2	3 7	+0 8

weekly periods In Tables 2 and 2-*a* the data are also given as totals or averages of weekly periods but in 4 instances for convenient comparison these represent expansions of shorter periods as indicated

In Tables 1 and 1 *a* is depicted a cycle of protein depletion and partial repletion all occurring during the first 4 weeks From an initial level of 5 81 per cent the plasma protein was reduced by fasting and plasmapheresis and then by low protein diet and more plasmapheresis to 3 79 per cent by the end of 2 weeks Only a small fraction (at most 5 gm) of the fifty-odd grams of plasma protein removed can have come from materials in the 15 gm of crude vitamin accessories The bulk of this protein represents a reduction in the reserve store of protein (at least 35 gm) and the balance is yielded by a reduction in the mass of plasma protein in the circulation (about 10 gm) No steady level of hypoproteinemia was attempted at the close of the second period, so that it cannot be clearly demonstrated that

the reserve store was in nice balance with the briefly maintained low plasma concentration of protein Rather the plasma level was allowed to rise during the 3rd week and reached 4 71 gm per cent on the last day, while a liver basal diet was being instituted and plasmapheresis was limited It was desired to keep the level between 4 6 and 4 7 per cent so that more protein was removed during the fourth period The level promptly fell to 4 38 per cent It became apparent that 15 gm plasma protein could not be removed if this higher level were to be maintained Note that 16 gm appears to be the amount which can be removed weekly in maintaining the level near 4 per cent (periods 10, 11, 12, and 18)

In periods 5 to 8 plasmapheresis was decreased The removal of 6 9 gm protein during period 7 dropped the level of plasma protein from 4 64 at the end of period 6 to 4 41 at the end of period 7 And with complete cessation of plasmapheresis during period 8 the concentration rose only to 4 71 gm per cent This rise of 0 3 gm per 100 ml plasma in plasma volumes as measured on those 2 days of 489 ml and 513 ml represents an increase in the mass of circulating protein of only 2 6 gm If 16 gm of new plasma protein were produced during this 8th week, a quantity within the capacity of this dog when maintained at a level of 4 per cent, then at least 13 gm of it has moved out of the circulation We have no means of determining whether new plasma protein was converted into tissue protein or whether the tissue protein was formed directly from digestion products We do believe that approximately this quantity of protein was formed in or stored in the tissues, in that the urinary nitrogen figures are comparable after the third period whether the plasma protein concentration is high or low

During the 9th week only 7 gm plasma protein were removed above the basal output of 16 gm In a previous experiment (5) it appeared that protein produced during 2 weeks of rest from plasmapheresis was almost quantitatively removed in again establishing a steady level at 4 per cent In this experiment 38 gm plasma protein were removed during periods 4 to 8 and at 16 gm per period a total of 80 gm might have been produced, yet only 7 gm of the 42 gm difference were removed It is reasonable to argue that this longer period allowed the body cells to modify some of the reserve proteins toward integral or immobilized protein There was also a gain in weight

It is to be emphasized that Tables 1 and 1-*a* approximate a perfect metabolic experiment After the initial periods 1 and 2, the dog was in total nitrogen balance, in weight equilibrium (actually a slight gain 0 3 kilos in 10 weeks), in perfect clinical condition with 100 per cent intake of a rather

unpalatable food mixture These factors are essential for a clean cut experiment and the conclusions have sound basis of fact In contrast Tables 2 and 2 *a* show in period 17 that refusal to eat will terminate a promising experiment

Tables 2 and 2 *a* present another experiment in the search for the specific construction materials in plasma protein synthesis In our previous experiments (6, 4) amino acids and incomplete proteins plus amino acids have been added to already adequate basal diets In periods 14 and 15 gelatin, 20 gm per day, and the mixture called "amino acids A" were added to a low protein diet consisting of sugar, starch, fat, salts, and accessories (see Experimental History, dog 36 196)

Amino acids A, per day	
	gm
l-cystine	1 5
dl isoleucine	1 0
l tyrosine	1 0
d valine	0 5
dl methionine	0 3
l tryptophane	0 3
ethyl β hydroxybutyrate	1 0

In the preliminary 2 days, period 14, only samples of blood for chemistry were removed but in the test period 15, more than 18 gm plasma protein were removed and the average concentration was significantly higher than in the control period 13 This experiment indicates a definite production of new plasma protein from materials contained in the gelatin, the amino acids, and the small amounts of protein of the yeast and the liver fraction in the basal diet This mixture of proteins and amino acids yielded only 14 per cent return as plasma protein, and less if the 32 2 gm of amino acids were considered as protein, as compared with a 19 per cent return of plasma protein from the protein of the liver basal diet

The after period 16 is comparable to period 13 on the low protein diet The mixture of period 17 was refused by the dog on the 3rd day and to avoid difficulties the animal was returned immediately to the liver basal diet, responding with a normal basal production of plasma protein, period 18

Amino acids B per day	
	gm
l-cystine	1 5
l tryptophane	0 3
dl methionine	0 3

steady state of hypoproteinemia and a uniform plasma protein production on a basal low protein diet These dogs are clinically normal but their resistance to infection is distinctly below normal Introduction of variables into this standardized existence gives information relative to plasma protein production

Plasma protein production under these conditions with a plasma protein concentration of 3 5 to 4 2 gm per cent is relatively constant As the plasma protein concentration rises the plasma protein removed falls rapidly (Table 1) At 4 6 gm per cent the protein removed is less than 50 per cent of the amount removed at a plasma protein level of 4 0 gm per cent

Cystine appears to be an important amino acid for plasma protein formation This shows in Table 2 and is supported by data coming from published experiments

These experiments related to the factors which control *plasma protein production* bear on the problems of *shock*, *hemorrhage*, and *protein wastage* and their treatment by *plasma injections* which hold the attention of surgeons and physiologists at the moment

Again we would emphasize the fluidity of body protein including plasma protein—an ebb and flow between protein depots and plasma protein—a "dynamic equilibrium" of body protein A discussion of the passage of large protein molecules through cell borders is submitted

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STUDIES ON INFLUENZA VIRUS

THE COMPLEMENT-FIXING ANTIGEN OF INFLUENZA A AND SWINE INFLUENZA VIRUSES

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Complement fixation studies on influenza A virus (1) infections have been conducted chiefly with human sera and antigens derived from influenza mouse lungs The use of this test to assay ferret immune sera, however, seems to have been hampered by the occurrence of non specific or heterophile reactions (2) when such sera were tested against mouse lung suspensions Since complement fixation offered a possible means of study ing further the antigenic differences between strains of influenza A virus, a problem of considerable importance in view of the recent evidence that clinical influenza is a disease of diverse etiology (1, 3*a*, *b*), experiments were conducted to evolve a satisfactory test with the ultimate end of applying it to this problem

Material and Methods

Source of Antigen.—Complement fixing antigens were prepared from the lungs of mice and ferrets and from the chorioallantoic and amniotic membranes of the developing chick embryo inoculated with influenza virus One strain S 1976, of swine influenza virus and the PR8 and W S strains of influenza A virus were used

Albino Swiss mice were inoculated intranasally under ether anesthesia with 0 05 cc. of a 0 1 per cent suspension of infected mouse lung 3 days after inoculation the animals were killed with chloroform and the lungs were removed and pooled Ferrets were inoculated intranasally with 1 0 cc of a 0 1 per cent suspension of virus-containing mouse or ferret lung and the lungs were removed at various intervals as subsequently noted

The procedure described by Nigg Crowley and Wilson (4) was used to obtain antigen from the developing chick embryo 0 1 cc of a 10 per cent suspension of infected chick tissue was inoculated between the yolk sac and the chorioallantoic membrane of 10 to 12 day old chick embryos After 2 days incubation at 37 C the chorioallantoic and amniotic membranes were removed washed in saline and pooled

Preparation of the Antigen.—Tissues (mouse lung ferret lung or chick embryo membranes) containing influenza virus were weighed ground with alundum and made into a 10 per cent suspension with saline The suspension was centrifuged at 2000 R P M for 30 minutes and the turbid supernate was drawn off, distributed into celluloid tubes

refrigerator was employed to facilitate any fixation which might occur, none of the four preparations, however, displayed even the slightest evidence that any complement-deviating antigen was present

Aside from whether the virus particle itself or some constituent or metabolic product of it is concerned in the complement fixation reaction, the injection into rabbits of tissue suspensions containing the antigen should give rise to specific complement-deviating antibodies

The remainder of the four ferret lung suspensions was injected into rabbits, two animals for each preparation Each rabbit received five intraperitoneal injections of 3 0 cc each, spaced 4 to 5 days apart Between injections the material was stored at −18°C and thawed rapidly when required for use, the tubes were refrozen as soon as the requisite amount had been withdrawn The animals were bled by cardiac puncture 10 days after the last injection These sera were titered against a PR8 mouse lung antigen (1 hour primary incubation) for their complement-fixing activity, and, to rule out the occurrence of non specific fixation, also against a control antigen prepared from normal mouse lungs Subsequently these sera were assayed against 30 fifty per cent mortality doses of PR8 mouse lung virus for their neutralizing capacity (9) Results obtained with four of the sera are presented in Table I

As shown in Table I, all four ferret lung suspensions appeared to be devoid of complement-fixing antigen when tested directly in the fixation test, yet on injection into rabbits all induced the formation of complement-deviating antibodies, even the lung removed 24 hours after inoculation of virus stimulated a marked response in the rabbit That these antibodies were specific for the soluble antigen was indicated by the failure of the sera to fix complement in the presence of normal mouse lung specimens Further, in view of the large amount of material used for immunization, the neutralizing titer of the rabbit sera was surprisingly low as compared with the complement-fixing titer If it is assumed that the unusually low neutralizing antibody titers were a direct consequence of a relative paucity of virus in the inocula, then it is justifiable also to assume that the relatively high complement-binding titers were due to the stimulus provided by the complement-fixing antigen itself Since the latter is a soluble antigen, separable and distinct from the virus (6, 8), it appears probable that the disproportionate amounts of the two antibodies produced in the rabbit reflected the concentration of the corresponding two antigens in the inocula, it will be recalled that the inocula were centrifuged at 13,000 R P M for 30 minutes, a procedure which removes approximately 90 per cent of the virus but affects the concentration of the soluble antigen to a lesser extent (8), in addition, storage at −18°C , while useful to preserve the soluble antigen, is not conducive to survival of the virus

From these data it was concluded that the antigen was actually present in infected ferret lungs, and that with careful and accurate adjustment of the reagents in the test, it might be possible to demonstrate the occurrence of specific fixation, although obviously the system could not be made too sensitive if non specific reactions were to be avoided

TABLE I

Results of Complement Fixation Tests with Ferret Lung Antigens and Convalescent Human Serum as Well as with Mouse Lung Antigens and Serum of Rabbits Inoculated with Ferret Lung Antigens

Ferret	Interval between intra nasal inoculation and removal of lung	Complement fixation test: Ferret lung antigen dilution	Complement fixation test: Dilution of serum: Pooled human convalescent 1 2	1 4	1 8	Rabbit immunized with ferret lung	Rabbit immune serum: Neutralizing capacity against PR8 strain	Complement fixation test†: PR8 mouse lung antigen dilution	Dilution of serum 1 4	1 8	1 16	1 32	Normal mouse lung antigen dilution	Dilution of serum 1 4	1 8	1 16
	hrs						*log*									
1	24	1 2	–	–	–	1	1 50	1 2	++++	++++	++	+	1 2	–	–	–
		1 4	–	–	–			1 4	++++	++++	++	+	1 4	–	–	–
		1 8	–	–	–			1 8	++++	+++	++	+				
								1 16	+++	++	+	–				
2	48	1 2	–	–	–	2	4 80	1 2	++++	++++	+++	++	1 2	++	–	–
		1 4	–	–	–			1 4	++++	+++	+++	+	1 4	–	–	–
		1 8	–	–	–			1 8	++++	++++	++	+				
								1 16	+++	++	++	+				
3	72	1 2	–	–	–	3	4 80	1 2	++++	++++	+++	++	1 2	++	–	–
		1 4	–	–	–			1 4	++++	++++	++	+	1 4	–	–	–
		1 8	–	–	–			1 8	+++	+++	++	+				
								1 16	+++	++	++	–				
4	96	1 2	–	–	–	4	4 10	1 2	++++	++++	++++	+++	1 2	++	–	–
		1 4	–	–	–			1 4	++++	++++	+++	+	1 4	–	–	–
		1 8	–	–	–			1 8	++++	++++	+++	+				
								1 16	++++	+++	++	+				

* Inoculation = 1000 M.L.D. of ferret passage PR8 strain

† Primary incubation period of 1 hour at 37 C

Ferret lung antigens were prepared as before and titered in serial twofold dilutions against falling twofold dilutions of ferret sera It soon became evident that (*a*) ferret lungs, weakly antigenic at best, varied in antigen content (*b*) overnight fixation in the cold was necessary, and (*c*) accurate quantitation of reagents was required, especially in the case of complement, small excesses of which were frequently sufficient to obliterate the weak positive reactions obtained Two and one half *exact* units were found to give the best results

Table II presents the results obtained with two ferret sera titrated against

TABLE III

Comparison of the Ability of Influenza Virus Antigens Derived from Three Animal Species to Fix Complement with Ferret Sera

Antigen		Serum																					
		Ferret 7								Ferret 8								Ferret 9					
		Preinfection				Convalescent				Preinfection				Convalescent				Preinfection				Convalescent			
Source	Dilution	1 4	1 8	1 16	1 32	1 4	1 8	1 16	1 32	1 4	1 8	1 16	1 32	1 4	1 8	1 16	1 32	1 4	1 8	1 16	1 32	1 4	1 8	1 16	1 32
Mouse lung	1 2	+	−	−	−	++++	++++	+++	+++	+	−	−	−	++++	++++	++++	++++	+	−	−	−	++++	++++	++++	+++
	1 4	±	−	−	−	++++	++++	++	±	~	−	−	−	++++	++++	++++	++++	±	−	−	−	++++	++++	++++	++
	1 8	−	−	−	−	++++	++++	++++	±	~	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	++++
	1 16	−	−	−	−	++++	++++	++++	+++	~	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	++++
Chick membrane	1 2	++	++	++	+	++++	++++	++++	++++	+	±	−	−	++++	++++	++++	++++	+++	+	+	−	++++	++++	++++	++++
	1 4	+	−	−	−	++++	++++	++++	++++	~	−	−	−	++++	++++	++++	++++	+	±	−	−	++++	++++	++++	++++
	1 8	−	−	−	−	++++	++++	++++	+++	~	−	−	−	++++	++++	++++	++++	±	−	−	−	++++	++++	++++	++++
	1 16	−	−	−	−	++++	++++	++++	++	~	−	−	−	++	++	++	++	−	−	−	−	++++	++++	+++	++
Ferret lung	1 2	±	−	−	−	++++	++++	++++	++++	~	−	−	−	++++	++++	++++	+++	−	−	−	−	++++	++++	++++	++++
	1 4	−	−	−	−	++	++	++	++	~	−	−	−	+	+	+	+	−	−	−	−	+++	+++	+++	+++
	1 8	−	−	−	−	+	+	−	−	~	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
	1 16	−	−	−	−	+	−	−	−	~	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

antigen is also included for comparison, all three antigens were tested simultaneously with the same reagents so that the experimental conditions were practically identical It will be noted that the mouse and chick antigens possessed about the same degree of complement fixing activity and that both were definitely superior to the ferret antigen

Study of Some of the Factors Possibly Involved in Non-Specific Complement Fixation with Ferret Sera—Since non specific fixation was occasionally encountered, it was thought of interest to examine some of the factors which might play a part in such reactions Since mouse lung was known to contain heterophile antigen, it appeared possible that this might be responsible for non specific fixation occurring when ferret sera were tested with influenza mouse lung suspensions

Ferret sera were examined for the presence of heterophile antibodies by the method described by Paul and Bunnell (10) Sera were inactivated at 56 C for 30 minutes, and serial twofold dilutions in saline from 1 4 to 1 256 were prepared To 0 5 cc of the serum dilution was added 0 5 cc of 1 30 guinea pig complement followed by 0 5 cc of 2 per cent sheep cells (The cells were washed three times packed and stored at 4°C for from 4 to 7 days before use) 1 cc of saline was added to bring the volume to 2 5 cc. The mixtures were well shaken, incubated for 1 hour at 37 C and the extent of hemolysis was recorded ++++ was used to designate complete lysis of the cells and − to indicate absence of hemolysis Correspondingly, intermediate degrees of lysis were recorded by +, ++, and +++

Table IV shows the concentration of heterophile antibody in five ferret and five human sera chosen at random The lytic action of the ferret sera was quite marked and, unlike human sera, practically all ferret sera tested exerted a strong lytic action on sheep cells Ferret sera, therefore, contain large amounts of heterophile antibody which was thought to be responsible, in part at least, for some of the non specific fixation reactions which had been encountered Hence, attempts were made to remove the heterophile antibodies from ferret sera

The technique of Davidsohn (11) with some modifications, was employed Guinea pig kidneys were rinsed with saline finely minced with scissors, and washed repeatedly with saline until the washings appeared free of gross blood The tissue was then ground without abrasive and made into a 20 per cent suspension in saline Sheep and beef red cells were washed three times in saline then well packed by centrifugation and taken up in four volumes of saline to make 20 per cent suspensions

The three suspensions were boiled for 1 hour with frequent stirring small additions of distilled water were made at intervals to prevent drying of the tissues When cool the supernates were poured off and saved while the sediments were triturated in a mortar these were then taken up in the original supernates, and distilled water was added to replace that lost by evaporation

hand, the PR8 and W S strains, the so called "intermediate and specific strains," are seemingly unique in that each occupies a niche of its own (12, 13) in the classification scheme The results of cross immunity tests have tended to confirm these findings (13, 14)

While such tests have demonstrated that strains of the virus may differ markedly one from another, they have yielded no information as to what portion of the virus particle endows it with its strain specific properties In conducting epidemiologic surveys with the use of the complement fixation test, antigens prepared from widely dissimilar strains have been employed (*e g* , W S by the English workers, Melbourne by the Australian, PR8 by the American, etc) with equally good results As a consequence the complement-fixing antigen has come to be considered generally as "group specific " Such an assumption has not been entirely warranted on the basis of the data available since human sera also possess the capacity to neutralize antigenically diverse strains to about the same extent, and hence would not be expected to reveal serologic differences in the complement-fixing antigens of these strains Ferrets, on the other hand, can be studied after a single attack of the disease and sera from these animals (13) can be shown by neutralization tests to reflect the differences in the antigenic structures of the infecting strains The use of ferret serum, therefore, appeared to offer a means of determining whether the strain specific properties of the virus were a function of the infectious moiety or of the soluble (complement-fixing) antigen

To determine whether serologic differences between strains of influenza A virus were demonstrable by the complement fixation test, five strains of virus isolated from four different epidemics in New York State in 1939 (9) were chosen

Ferrets were inoculated intranasally with each of these strains and bled 10 to 12 days later Similar sera from ferrets inoculated with the PR8 or W S strains known to be dissimilar antigenically by reciprocal cross neutralization and cross immunity tests as mentioned above were included for comparative purposes A PR8 mouse lung antigen was used in falling twofold dilutions with similar dilutions of each of these sera Primary incubation was carried out at 37°C for 1 hour followed by 4°C overnight Neutralization tests, as previously described (9), were done to determine the neutralizing potency of the sera and since, for comparative purposes, the concentration of this antibody is more exactly expressed as neutralizing capacity (15, 16) than as the serum dilution end point, the neutralizing capacities of these sera will be presented here

The data in Table VI show that no remarkable qualitative differences were demonstrable in the complement-fixing titers of these influenza A virus antisera In a general way, the apparent quantitative differences

seem to be related to the neutralizing capacities of the sera It might, however, be possible that the rough correspondence of the complement-

TABLE VI

Results of Cross Complement Fixation Tests with the PR8 Strain and Convalescent Ferret Sera against Various Strains of Influenza A Virus

Ferret number	Recovered from infection with	Neutralizing capacity of serum against PR8	Dilution of PR8 antigen	Dilution of convalescent ferret serum			
				1 4	1 8	1 16	1 32
	strain	*log*					
15	149	7 27	1 2	++++	++++	++++	+++
			1 4	++++	++++*	++	+
			1 8	++++	++++	+	−
16	188	6 62	1 2	++++	++++	++++	++
			1 4	++++	++++	++++	++
			1 8	++++	++++	++++	+
17	236	6 19	1 2	++++	++++	++++	++++
			1 4	++++	++++	++++	+++
			1 8	++++	++++	++	+
18	273	5 73	1 2	++++	++++	+++	++
			1 4	++++	++++	++	−
			1 8	++++	+++	+	−
19	399	4 80	1 2	++++	++++	++	−
			1 4	++++	++	−	−
			1 8	++++	+	−	−
20	W S	7 57	1 2	++++	++++	++++	+++
			1 4	++++	++++	++++	++
			1 8	++++	++++	++++	++++
			1 16	++++	++++	++++	++++
21	PR8	6 80	1 2	++++	++++	++++	+++
			1 4	++++	++++	++++	++
			1 8	++++	++++	++++	++++
			1 16	++++	++++	++++	++++

Sera inactivated at 56 C. for 30 minutes

Primary incubation period was 1 hour at 37 C and overnight at 4 C

* Mouse lung antigen

fixing titers and neutralizing capacities resulted from the fact that all the sera except the PR8 serum were tested against a heterologous and not a homologous strain of virus and soluble antigen

Ferret immune sera against the PR8 and W S strains of influenza A virus and a strain (S 1976) of swine influenza virus were obtained by cardiac puncture 10 to 12 days after

TABLE VII

Results of Cross Complement Fixation Tests with Swine Influenza Virus, the W S and PR8 Strains of Influenza A Virus, and Ferret Antisera

Ferret number	Influenza virus inoculated	Serum specimen tested	Neutralizing capacity of serum against*			Dilution of antigen	Dilution of serum tested against													
							Swine antigen					W S antigen					PR8 antigen				
			Swine	W S	PR8		1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64
			log	*log*	*log*																
22	Swine	Preinfection	0	–	–	1 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Convalescent	4 67	1 93	1 93	1 2	++++	++	+	–	–	++++	++	–	–	–	++++	++	–	–	–
						1 4	+++	+	–	–	–	++++	+++	–	–	–	++	+	–	–	–
						1 8	++	–	–	–	–	++++	–	–	–	–	++	–	–	–	–
						1 16	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
23	Swine	Preinfection	0	–	–	1 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Convalescent	5 42	3 02	2 17	1 2	++++	++++	+++	–	–	++++	++++	+	–	–	++++	++++	++	–	–
						1 4	++++	++	+	–	–	++++	+++	+	–	–	++++	++++	–	–	–
						1 8	–	–	–	–	–	+	–	–	–	–	±	–	–	–	–
						1 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	Swine	Convalescent	5 42	3 22	2 77	1 2	++++	++++	++++	+	–	++++	++++	++	–	–	++++	++++	+++	–	–
						1 4	++++	++++	++++	–	–	++++	++++	++	–	–	++++	++++	+	–	–
						1 8	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–
						1 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	W S	Preinfection	–	0	0	1 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
						1 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Convalescent	3 39	6 96	6 57	1 2	++++	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
						1 4	++++	++++	+++	+	–	++++	++++	++++	+++	++	++++	++++	++++	+++	+++
						1 8	–	–	–	–	–	+++	+++	++	+	–	+++	++	++	–	–
						1 16	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

26	W S	Convalescent	—	6 55	5 80	1 2	+++	—	—	—	—	++++	+++	+++	—	—	++++	++++	—	—	—
						1 4	++	—	—	—	—	++++	+	—	—	—	++++	++	—	—	—
						1 8	±	—	—	—	—	+	—	—	—	—	+	—	—	—	—
						1 16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
27	PR8	Convalescent	2 90	—	6 55	1 2	++++	++++	++++	—	—	++++	++++	++++	++	—	++++	++++	++++	++	—
						1 4	++++	++	+	—	—	++++	++++	++++	—	—	++++	++++	+++	++	—
						1 8	++	—	—	—	—	++++	++	++	—	—	+++	+	—	—	—
						1 16	—	—	—	—	—	++	—	—	—	—	±	—	—	—	—
28	PR8	Convalescent	—	—	6 55	1 2	++++	++++	++	—	—	++++	++++	++++	++++	—	++++	++++	++++	++++	—
						1 4	++++	++	±	—	—	++++	++++	++++	++	—	++++	++++	++++	+++	—
						1 8	++	—	—	—	—	++++	+++	++	—	—	++++	+++	++	—	—
						1 16	—	—	—	—	—	+	—	—	—	—	+	—	—	—	—

* 0 = no neutralizing antibodies

— = neutralizing capacity not determined

inoculation of these strains The swine virus was included because it is so distantly related antigenically to the PR8 and W S strains (12–14)

Mouse lung antigens were prepared from these three strains of virus, and the ferret sera were tested for complement-fixing and neutralizing capacities as described in the section above The results are summarized in Table VII

An analysis of Table VII brings out a number of interesting facts It will be noted that the swine antisera neutralized large quantities of swine virus, much less of the W S strain, and still less of the PR8 strain The serum of ferret 24, for example, was capable of neutralizing 262,000 fifty per cent mortality doses of the homologous (swine) virus, but only 1660 such doses of the W S strain and 588 of the PR8 strain The antigen of the swine virus, however, fixed complement with the heterologous W S and PR8 antisera, almost equally as well as with its own homologous antisera Insufficient quantities of the particular PR8 antisera studied were available to permit complete neutralization experiments, but in view of what is already known (12, 13) concerning these three strains, the results should have been comparable to those obtained with the W S strain In the case of ferret serum 25, as an example, it was found that this serum neutralized 9,120,000 fifty per cent mortality doses of the homologous strain (W S), 3,710,000 such doses of the heterologous but related PR8 strain, but only 2180 doses of the distantly related swine virus By the complement fixation test, on the other hand, no differences in the titers of the PR8 and W S antisera were demonstrable whether these were tested against the homologous or heterologous antigen When tested against the swine antigen, however, these influenza A virus antisera gave distinctly lower levels of fixation

It is evident that the titers obtained in cross complement fixation tests were unrelated to the capacities of the sera to neutralize markedly heterologous strains The PR8 and W S strains, while related, are known to be different antigenically by cross neutralization tests, by cross complement fixation tests, however, their soluble antigens appeared to be so similar that it proved impossible to distinguish one strain from the other An antigenic component present in the swine soluble antigen appeared to be present also in the PR8 and W S antigens as each of the corresponding antisera fixed complement to a like degree with the swine complement-fixing antigen This was also borne out by the finding that swine antisera in the presence of swine, PR8, or W S antigens fixed complement to the same extent

DISCUSSION

In studying the complement fixing antigen of influenza virus, the use of ferrets appeared to possess several advantages These animals, susceptible to infection with influenza viruses, could be made to pass through an attack of the disease under controlled conditions, which obviously does not hold true for man Also antibodies arising as a result of the actual disease were thought to approximate more nearly natural conditions than would antibodies induced by artificial immunization

Because of the difficulties reported (2) to occur with complement fixation tests in which ferret serum was used with influenza antigens obtained from animal species (*e g*, the mouse) possessing heterophile antigen, it was essential to investigate this problem first An attempt was made to employ infected ferret lung as a source of antigen, but the use of this tissue had to be abandoned when it became evident that even under the best conditions its antigen content was very low and not infrequently undemonstrable A point of interest emerged when infected ferret lungs, containing no demonstrable complement fixing antigen, were injected in large amounts into rabbits, these animals responded by the production of antisera with a surprisingly low virus neutralizing capacity but a comparatively good complement fixing titer, for example, the serum of rabbit 1, which failed in a dilution of 1 2 to neutralize 30 fifty per cent mortality doses of virus, nevertheless gave good fixation of complement As the lung suspensions had been centrifuged at high speed, which removes considerable virus, and subsequently had been stored at −18°C and thereafter frequently thawed and refrozen, a procedure favoring inactivation of the virus, the marked differences in the titers of the neutralizing and complement-fixing antibodies suggest that these arose from stimuli afforded by two different and distinct antigens

The use of infected mouse lungs or developing chick embryo membranes as a source of antigen was attended by a high proportion of non specific fixations, and some difficulty was encountered by the absence of specific complement fixation in the presence of ferret sera which, *a priori*, would have been expected to react positively When titration of the complement was made as quantitatively exact as possible, practically all the non specific reactions disappeared Full units of complement were not used, as even small excesses of this agent were sufficient to abolish otherwise positive, specific fixation These points were found to be of the utmost importance if success was to be achieved in fixation systems utilizing ferret sera

That heterophile antibodies are present in ferret sera was demonstrated

inoculation of these strains The swine virus was included because it is so distantly related antigenically to the PR8 and W S strains (12–14)

Mouse lung antigens were prepared from these three strains of virus, and the ferret sera were tested for complement-fixing and neutralizing capacities as described in the section above The results are summarized in Table VII

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similar, if not identical They indicated also that the soluble antigen of swine virus possessed components present in the antigens of the human strains of virus

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THE QUANTITY OF IRRADIATED NON-VIRULENT RABIES VIRUS REQUIRED TO IMMUNIZE MICE AND DOGS

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In a previous paper we pointed out that 1 5 cc of irradiated tissue culture virus successfully immunizes mice against a subsequent intracerebral or intramuscular test infection (1) It soon developed, however, that extremely large volumes of the irradiated culture virus were needed to immunize dogs Quantitative studies were undertaken, therefore, to determine the amounts of vaccine necessary for mice and dogs in terms of mouse intracerebral lethal doses

Minimum Volume and Virus Content of Culture Virus Required for Immunization of Mice

The first experiments dealt with the minimum dose of tissue culture vaccine capable of immunizing mice In the previous paper (1), we stated that the culture virus usually titred 0 03 cc of the 10^{-3} dilution in 4 to 6 weeks old mice and was therefore said to contain 33,000 mouse intracerebral lethal doses per cc 1 5 cc , or approximately 50,000 doses, properly irradiated, immunized the mice More recently we have adopted as a standard for titration 3 weeks old mice rather than those aged 4 weeks or more In these younger mice the culture virus often titres one dilution higher, 0 03 cc of the 10^{-4} dilution

Repeated experiments to determine the least amount of irradiated tissue culture virus[1] necessary to immunize mice indicated that 50,000 mouse doses, properly irradiated, gave good protection, whereas less than this amount, although sometimes effective, was not consistently so The following experiments illustrate the type of result obtained

Experiment 1 —Tissue culture virus, Pasteur strain[2] prepared as previously described (1), was distributed in three quartz flasks One was irradiated 20 minutes and then

[1] We are indebted to Dr George I Lavin for irradiating the preparations used in these experiments

[2] This strain was kindly sent by Dr Pierre Lépine of the Pasteur Institute Paris, and has been passaged in mice in our laboratory

tested for virulence, the second 30 minutes and tested, and the third 40 minutes and tested for virulence by inoculating 0 03 cc intracerebrally into each of five mice The 30 minute irradiated virus was then injected as a vaccine intraperitoneally into 30 day old Swiss mice in the following manner Group 1 received 1 cc in a single injection, group 2, an injection of 0 5 cc and 2 days later a second injection of 0 5 cc , and group 3 received 0 2 cc every other day for five injections Group 4 received 0 5 cc in a single injection, group 5, 0 25 cc , and 2 days later a second injection of 0 25 cc Group 6 received 0 1 cc daily for 3 days and finally, group 7 received no vaccine

3 weeks after commencing vaccination the mice were tested for immunity by injecting them into the gastrocnemius muscle with 0 01 cc of virulent rabies virus in serial two-fold dilutions, four mice being employed for each dilution All mice were observed 4 weeks for signs of rabies

The virulence of the virus before irradiation (Table I) proved to be 0 03 cc of the 10^{-4} dilution or better Hence the material is said to contain 330,000 mouse doses per cc or more Following irradiation for 20, 30, or 40 minutes, the material injected intracerebrally into mice failed to kill In the immunity test, the non-vaccinated mice titred 1 to 1,800 according to the method of Reed and Muench (2), whereas the batches of vaccinated mice withstood 23 5 and 47+ times this amount, indicating a strong immunizing potency of the vaccine In this experiment, 111,000 or more doses of irradiated vaccine given by any of the above methods immunized effectively

Experiment 2 —Tissue culture virus was prepared, irradiated, and tested for virulence as in Experiment 1 0 5 cc of the 20 minute irradiated virus was injected as a vaccine intraperitoneally into a batch of 30 day old Swiss mice The 30 and 40 minute preparations were likewise injected into a second and third batch A fourth batch received three injections of 0 5 cc of the 30 minute irradiated vaccine and the fifth batch was left unvaccinated as controls 3 weeks after commencing vaccination the vaccinated and unvaccinated mice were given a test dose of virulent virus intramuscularly, as described in Experiment 1, and observed 4 weeks for signs of rabies

The results of this test are shown in Table II The virulence of the virus before irradiation proved to be 0 03 cc of the 10^{-3} dilution Hence the material is said to contain 33,000 mouse doses per cc Following irradiation three of the five mice injected with the 20 minute material succumbed to rabies, two of the five injected with the 30 minute, and none injected with the 40 minute irradiated virus Only this latter preparation was regarded, therefore, as non-virulent In the immunity test the challenge virus in the non-vaccinated mice titred 1 to 1,280 The mice vaccinated with 16,500 doses withstood three times and those vaccinated with 49,500 doses withstood thirty-one times as much virus, indicating a strong immunity In passing, it may be noted also that, even though most of the vaccine given in this experiment contained a trace of virulent virus, it was too small in quantity to affect the immunizing potency of the material (1)

TABLE I

Immunisation of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus

Virulence before irradiation (0 03 cc in dilutions intracerebrally) 10^{-} – 3/4* 10^{-3} – 2/4 10^{-4} – 2/4

" following (0 03 cc. undiluted) 20 minutes – 0/5 30 minutes – 0/5

Immunity of Vaccinated Mice

Dose of vaccine		Mortality of mice given test virus intra muscularly (0 01 cc) in dilutions							Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
		1/80	1/160	1/320	1/640	1/1 280	1/2 560	1/5 120		
Group 1	1 cc 1 dose (330 000 M.D ‡)	0/4	0/4	0/4	0/4	0/4	—	—	<80	47+
2	0.5 cc 2 doses	0/4	0/4	0/4	0/4	0/4	—	—	<80	47+
3	0.2 cc 5	0/4	0/4	0/4	0/4	0/4	—	—	<80	47+
4	0.5 cc 1 dose (165 000 M.D)	2/4	0/4	0/4	0/4	0/4	—	—	80	23 5
5	0.25 cc 2 doses	0/4	0/4	0/4	0/4	0/4	—	—	80	23 5
6	0 1 cc 3 (111 000)	0/4	1/4	0/4	0/4	0/4	—	—	<80	47+
7	No vaccine	—	2/4	4/4	3/4	3/4	3/4	0/4	1 800	

* 3/4 = 3 of 4 mice injected died of rabies † Estimated by method of Reed and Muench (2)

‡ Mouse intracerebral lethal doses in vaccine prior to irradiation — = material not tested

TABLE II

Immunisation of Mice with Graded Doses of Irradiated Tissue Culture Pasteur Rabies Virus

Virulence before irradiation (0 03 cc. in dilutions intracerebrally) 10^{-2} – 3/3* 10^{-3} – 3/3 10^{-4} – 0/3

" following (0 03 cc undiluted) 20 minutes – 3/5 30 minutes – 2/5 40 minutes – 0/5

Immunity of Vaccinated Mice

Dose of vaccine		Mortality of mice given test virus intramuscularly (0.01 cc) in dilutions								Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
		1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5 120		
Group 1	0.5 cc 1 dose (16,500 M.D ‡) Irradiated 20 min	4/4	2/4	3/4	2/4	1/4	2/4	—	—	320	4 0
2	Same dose Irradiated 30 min.	4/4	3/4	3/4	3/4	2/4	0/4	—	—	416	3 1
3	Same dose Irradiated 40 min.	3/3	3/4	2/4	3/4	2/4	1/4	—	—	415	3 1
4	0.5 cc. 3 doses (49 500 M.D) Irradiated 30 min	0/4	1/4	0/4	0/4	0/4	0/4	—	—	<40	31 2
5	No vaccine	—	—	3/4	3/4	2/4	3/4	1/4	1/4	1 250	

Footnotes the same as in Table I

TABLE III

Immunisation of Mice with Graded Doses of Concentrated Irradiated Tissue Culture Pasteur Rabies Virus

Virulence before irradiation (0 03 cc. in dilutions intracerebrally) 10^{-2}–2/2* 10^{-3}–2/2 10^{-4}–1/2 10^{-5}–0/

" following (0 03 cc undiluted ") 45 minutes – 0/4

Immunity of Vaccinated Mice

Dose of vaccine		Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions						Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
		1/80	1/160	1/320	1/640	1/1 280			
Group 1	1.5 cc., 1 dose (49,500 M.D ‡)	0/4	0/4	0/4	0/4	—		<80	6.3+
2	0 15 cc 1 dose (49,500 M.D) concentrated 10 times	0/4	0/4	0/4	0/4	—		<80	6.3+
3	0 5 cc 1 dose (16 600 M.D)	3/4	2/4	1/4	0/4	—		160	3 2
4	0.5 cc 1 dose (166 000 M.D) concentrated 10 times.	—	0/2	0/3	0/3	—		<160	3 2+
5	0 01 cc 1 dose (3,300 M.D) co centrated 10 times	3/4	3/4	2/4	0/3	—		320	1 6
6	No vaccine	—	3/4	3/4	1/4	0/3		400	

Footnotes the same as in Table I

Protocols with further data are shown (Table III), supporting the conclusion that approximately 50,000 irradiated doses of culture virus are required to immunize mice

Minimum Volume and Virus Content of Culture Virus Required for Immunization of Dogs

The findings with mice led to a rough assay of the amount of vaccine required to immunize dogs The following experiment shows that beagle dogs of the sort used in previous work with vaccines (3), weighing 10 kilos, or about 500 times as much as 20 gm mice, were not immunized by 75 times but were immunized to some extent by 500 times the mouse dose of irradiated culture vaccine

Experiment 3 —Tissue culture virus was prepared, tested, irradiated 40 minutes, and tested again for virulence as in the previous tests Four beagle dogs, 4 to 6 months old and weighing 14 to 16 pounds, each received 450 cc of the irradiated vaccine intraperitoneally and another batch of four similar dogs each received 75 cc Five additional dogs were set aside as controls 3 weeks later, each received 0 25 cc of virulent virus diluted 1 to 200 (about one lethal dose (3)) into the neck muscles of the right and left sides They were observed for signs of rabies for 2½ months

The culture virus before irradiation titred 10^{-3} and hence contained 33,000 mouse doses per cc Following irradiation the vaccine was not virulent for the mice All five non-vaccinated dogs succumbed to the test virus on the 12th, 13th, 14th, 23rd, and 34th days and those receiving 75 cc on the 15th, 16th, 19th, and 25th days respectively, whereas of the four receiving 450 cc , one died of rabies on the 14th and one on the 63rd days, and the other two remained well

In view of the fact that dogs such as those used in our tests (3) needed as much as 500 cc of irradiated vaccine as prepared to become immune to the test virus, and hence of a possible relation between weight of animal and amount of vaccine required, attempts were made to secure a virus preparation with a greater number of mouse doses per cc Efforts to increase the titre of the culture virus have thus far been unsuccessful Concentration procedures, on the other hand, were encouraging as far as they were carried out

Experiment 4 —Tissue culture virus was centrifuged at 1,000 P P.M for 5 minutes, the supernatant drawn off, tested for virulence, irradiated 45 minutes with the mercury vapor lamp, and tested again for virulence 116 cc. of this relatively clear material, free of obvious tissue fragments, were evaporated to dryness at low temperature in reduced atmospheric pressure and then resuspended in distilled water to 11 6 cc , or one tenth of its original volume

Sixteen mice were injected intraperitoneally with 1 5 cc of the unconcentrated and sixteen mice with 0 15 cc. of the concentrated vaccine Moreover, sixteen were injected with 0 5 cc. of the unconcentrated and sixteen with 0 5 cc of the concentrated vaccine Sixteen mice were given 0 01 cc of the concentrated vaccine and sixteen mice remained unvaccinated 3 weeks later all mice were tested for immunity by injecting them in tramuscularly with 0 01 cc of virulent rabies virus in graded doses They were observed for signs of rabies for 4 weeks

The results of this experiment are shown in Table III The titre of the virus before irradiation was 0 03 cc of the 10^{-3} dilution Following irradiation it failed to kill the inoculated mice In the immunity test, the virus in unvaccinated mice titred 1 to 400 Mice dying from causes other than rabies are not included in the table All mice receiving 49,500 mouse doses in a 1 5 cc volume (group 1) and in the concentrated 0 15 cc volume (group 2) remained well Mice receiving 16,600 doses in 0 5 cc (group 3) showed little immunity, whereas those receiving 166,000 doses in 0 5 cc (group 4) remained well Finally, the mice receiving only 3,300 doses were not immunized 50,000 or more mouse doses, even in a ten times concentrated volume, remained capable of conferring a high grade immunity Similar tests have not been performed on dogs

Minimum Volume and Virus Content of Mouse Brain Tissue Virus Required for Immunization of Mice

Known sources of rabies virus other than tissue culture are limited largely to mammalian brain tissue Brain tissue has the advantage of affording the largest yield of virus and consequently is the standard source of vaccines, yet it has the concomitant disadvantage of accompanying the virus in concentrations of from 4 to 33 per cent Experiments were undertaken, therefore, to determine whether virus could be readily separated from brain tissue without loss of titre or immunizing potency

Comparative titrations on supernatants and sediments of centrifuged mouse brain preparations indicated that speeds of 3,000 R P.M for 20 minutes removed a large part of the brain tissue without appreciable loss of virus

Experiment 5 —The Pasteur strain of rabies virus was injected intracerebrally into the brains of four 3 weeks old W Swiss mice 6 days later when they became prostrate, the brains of two were removed, ground in a mortar, and made up to a 10 per cent suspension in water The material was then centrifuged 20 minutes at 3 000 R.P.M. in a Swedish angle centrifuge The supernatant was removed and the sediment brought back to its original volume in serum water Further dilutions of supernatant and sediment were made and titred intracerebrally in Swiss mice

The results of the titrations are shown in Table IV Both the supernatant and sediment titred as usual through the 10^{-7} dilution, indicating that as much titrable virus per cc was contained in the relatively clear supernatant as in the thick brain-tissue-containing sediment

Having learned that suspensions of mouse brain virus can be cleared by centrifugation without demonstrable loss of virulence, experiments were made to determine the relative immunizing potency of uncentrifuged and cleared vaccine

Experiment 6—9 cc of 33 per cent commercial chloroformized antirabies canine vaccine were diluted with 81 cc of horse serum water to make a 3 3 per cent suspension 40 cc of this suspension were used as a vaccine on one batch of mice The remaining 50 cc were centrifuged at about 2,500 R P M for 15 minutes, after which the supernatant became lightly opalescent and largely free of gross particles This supernatant was used as a vaccine on the second batch of mice

TABLE IV

Comparative Titre of Rabies Virus in Supernatant and Sediment of Brain Tissue Suspensions Centrifuged at 3,000 R.P M for 20 Minutes

Material	Fate of mice injected intracerebrally with virus suspensions (0 03 cc) in dilutions			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Supernatant	4/4	4/4	4/4	3/4
Sediment	4/4	4/4	2/4	3/4

Each mouse received a total of 1 cc of the given vaccine—one-half of each batch in a single dose and the other in five doses of 0 2 cc each 3 weeks later the four batches of vaccinated plus a fifth batch of unvaccinated mice were tested for their immunity to virulent virus injected in 0 01 cc doses in twofold dilutions into the gastrocnemius muscle

The results of the test (Table V) show that the test virus in unvaccinated mice titred 1 to 1,600 The mice given uncentrifuged material withstood at least twenty times and those given centrifuged material withstood five and twenty times this amount of virus respectively Apparently the centrifuged supernatant confers an immunity of the same order as that of uncentrifuged material

Further experiments showed that virus-containing supernatants prepared as above could be readily irradiated until virulence was destroyed and yet retain their immunizing potency

Experiment 7—Fifteen 3 weeks old W-Swiss mice were inoculated intracerebrally with 0 03 cc of the rabies Pasteur strain diluted 1 to 100 in serum water 6 days later the mice were prostrate, sacrificed, and their brains removed The brains were emulsified

with diluent to make a 10 per cent suspension This suspension was then titrated for virulence

18 cc were placed in a quartz flask, 9 cc plus 9 cc of diluent in a second flask and finally 1 5 cc. plus 13 5 cc. in a third flask to give final dilutions of 10 per cent 5 per cent and 1 per cent for irradiation Irradiation was then commenced and samples were

TABLE V

Immunization of Mice with Graded Doses of Centrifuged and Uncentrifuged Commercial 33 Per Cent Chloroformed Rabies Vaccine

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions							Titre of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5 120		
Group 1 1 cc. 1 dose	0/4†	0/4	1/4	0/4	0/4	—	—	<80	20+
2 0 2 cc 5 doses	0/4	0/4	0/4	0/4	0/4	—	—	<80	20+
3 1 cc 1 dose (super natant)	3/4	0/4	1/4	1/4	—	1/4	—	320	5
4 0 2 cc , 5 doses (super natant)	0/4	0/4	0/4	0/4	0/4	—	—	<80	20+
5 No vaccine	—	—	3/4	3/4	3/4	0/4	2/4	1 600	

* Estimated by method of Reed and Muench (2)

† 0/4 = none of 4 mice injected died of rabies

TABLE VI

Virulence of Mouse Brain Rabies Virus Following Irradiation with a Quart Mercury Vapor Lamp

Virulence of 10 per cent emulsion before irradiation 10^{-5} – 4/4* 10^{-6} – 4/4 10^{-7} – 0/4

10^{-6} – 0/4

Concentration	Fate of mice injected with undiluted material irradiated						
	20 min	30 min	40 min	50 min	60 min	70 min	80 min
per cent							
10	4/4	4/4	3/4	2/4	0/4	0/4	0/4
5	1/3	0/2	0/3	0/4	0/4	0/4	0/4
1	0/4	0/4	0/4	—	—	—	—

4/4 = 4 of 4 mice injected died of rabies

— = material not tested

withdrawn for virulence tests at 20 minutes and at 10 minute intervals thereafter for a total of 80 minutes

The results of the various virulence tests are shown in Table VI.

The virulence end point of the material before irradiation proved to be 0 03 cc of the 10^{-6} dilution The 10 per cent emulsion proved virulent after 50 minutes' but not longer irradiation, the 5 per cent after 20 but not

longer, and the 1 per cent not after 20 minutes' irradiation Both the 5 per cent and the 1 per cent preparations appeared to be as readily inactivated by ultraviolet light as the tissue culture materials

Experiment 8 —A 1 per cent suspension of rabies virus was prepared as above and tested for virulence A portion was set aside for inactivation with chloroform (Experiment 10) and a portion irradiated, and samples were tested for virulence at 10, 20, and 30 minutes The bulk of the vaccine after 30 minutes' irradiation was stored in the

TABLE VII

Immunization of Mice with Graded Doses of 1 Per Cent Irradiated and 1 Per Cent Chloroformized Mouse Brain Rabies Virus

Virulence before irradiation (0 03 cc in dilutions intracerebrally) 10^{-5} – 4/4* 10^{-6} – 4/4 10^{-7} – 3/3 10^{-8} – 2/4

" following " (0 03 cc undiluted ") 10 minutes – 5/5 20 minutes – 5/5 30 minutes – 2/10 30 plus 5 minutes – 0/5

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions									Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1 280	1/2 560		
Experiment 8 Irradiated vaccine											
Group 1 1 per cent 0.5 cc. (1 650 000 M.D ‡) irradiated	1/5	2/5	0/5	0/5	0/5	1/5	—	—	—	10	80
" 2 1 per cent 0 1 cc (330 000 M.D) irradiated	2/5	0/5	1/5	0/5	1/5	0/5	—	—	—	11	72
" 3 0 1 per cent 0 1 cc (33 000 M.D) irradiated	1/5	0/5	1/5	2/5	2/5	2/5	—	—	—	16	50
" 4 No vaccine	—	—	4/5	4/5	3/5	4/6	4/5	4/5	1/4	800	
Experiment 11 Chloroformized vaccine											
Group 1 1 per cent 0 1 cc (330 000 M.D)	2/4	2/4	0/4	3/5	1/5	1/5	—	—	—	31	26
2 0 1 per cent 0 1 cc (33 000 M.D)	4/5	3/5	2/5	2/5	2/5	1/5	—	—	—	48	16 5

Footnotes the same as in Table I

ice box to await the outcome of the virulence test The material before irradiation titred very high,—0 03 cc 10^{-8}—and following 30 minutes' irradiation was still fatal to two of ten injected mice Consequently it was given a second irradiation of 5 minutes, 8 days after the first This time the preparation proved non-virulent

The vaccine, then 13 days old, was given to mice intraperitoneally as follows One group received 0 5 cc of the 1 per cent suspension, a second group, 0 1 cc of the 1 per cent, and a third group, 0 1 cc of the 1 per cent diluted ten times to make a 0 1 per cent suspension 3 weeks later these mice, together with controls, were tested for resistance to an intramuscular injection of virulent virus administered as described above

The results are shown in Table VII According to the high titre of the virus before irradiation, the mice given 0 5 cc of the 1 per cent suspension

received at least 1,650,000 doses, those given 0 1 cc , 330,000 doses, and those given 0 1 cc of the 0 1 per cent suspension, 33,000 doses In the immunity test, challenge virus in the unvaccinated mice titred to an end point of 0 01 cc of 1 to 800 dilution, the mice vaccinated with 1,650,000 doses withstood eighty times as much virus, those vaccinated with 330,000 doses, seventy-two times, and with 33,000 doses, fifty times as much virus Apparently irradiated mouse brain virus gives clear cut protection in doses of 33,000

TABLE VIII

Immunization of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus

Virulence before irradiation (0 03 cc in dilutions intracerebrally) 10^{-5} – 4/4* 10^{-6} – 4/4 10^{-7} – 2/4 10^{-8} – 0/4

following (0 03 cc undiluted ') 35 minutes – 0/6

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intra muscularly (0.01 cc.) in dilutions					Titre† of virus in m ce	Difference in titre of virus in vaccinated and non vaccinated mice
	1/10	1/40	1/160	1/640	1/2,560		
No vaccine (A)	5/5	5/6	5/6	1/6	2/6	368	
(B)	5/5	5/5	4/5	1/5	1/5	384	
1 per cent 0 2 cc. (660 000 M.D †)	1/6	0/6	1/6	0/6	0/6	<10	37 5+
1 per cent 0 1 cc. (330 000 M.D)	3/5	3/5	0/5	1/5	0/5	40	9 6
0 1 per cent 0 1 cc. (33 000 M.D)	5/5	4/5	3/5	0/5	0/5	160	2 4
No vaccine (C)	5/7	4/5	2/5	0/5	—	40	
1 per cent 0 1 cc (330 000 M.D)	1/7	0/6	1/6	0/6	—	<10	4+

Footnotes the same as in Table I

Further tests showed that the critical dose for immunizing these mice was roughly in the neighborhood of 50,000, an amount similar to that found necessary for tissue culture virus

Experiment 9 —A 1 per cent suspension of rabies virus was prepared as above and tested for virulence It was then irradiated 35 minutes and tested again for virulence

15 days later the vaccine was given intraperitoneally to mice as follows One batch received 0 2 cc. of the 1 per cent vaccine and a second batch (A) was left unvaccinated as controls 5 days later, a second batch of mice was given 0 1 cc. of the vaccine, a third batch, 0 1 cc of the 1 per cent vaccine diluted ten times to make a 0 1 per cent preparation, and a fourth batch (B) was left unvaccinated as controls At the same time another batch was given 0 1 cc of the 1 per cent vaccine and a final batch (C) set aside as controls

3 weeks following vaccination, the mice were tested for immunity as follows The first two series prepared on the 15th and 20th days were tested intramuscularly with a virulent passage strain and the final series with a street strain which had received no laboratory passages

The results are shown in Table VIII According to the virulence tests, the vaccinated mice received 660,000, 330,000, and 33,000 doses of irradiated virus respectively (Table VIII) The immunity test showed that the test virus in the non-vaccinated mice (A and B) titred to an end point of 0 01 cc of the 1 to 368 and 384 dilutions respectively, the mice receiving 660,000 and 330,000 doses of vaccine withstood 37 5 and 9 6 times this amount respectively, indicating a considerable degree of immunity Mice vaccinated with 33,000 doses withstood 2 4 times as much virus as non-vaccinated mice Finally, street virus in non-vaccinated mice titred to the 1 40 dilution and mice vaccinated with 330,000 doses withstood at least four times as much virus, indicating a well marked immunity In short, 33,000 doses barely immunized the mice, whereas more than this amount gave good protection

Relative Immunizing Potency for Mice of Irradiated and Chloroformized Vaccines

The relative immunizing potencies of irradiated and chloroformized vaccines have been compared in five tests Three of these showed no striking differences, whereas two showed a superiority of irradiated vaccines

Experiment 10 —A 1 per cent suspension of mouse brain virus was divided into two parts, one was spun in a Swedish centrifuge at 3,000 R P M for 30 minutes and the other in a horizontal centrifuge at 500 R P M for 5 minutes The supernatants were both removed, titrated for virulence, exposed to ultraviolet light for 20 minutes, and 18 days later 0 25 cc was injected as a vaccine into mice every other day until four doses had been given 3 weeks later the vaccinated plus the control mice were tested for immunity to an intracerebral injection of virulent street virus

Both the Swedish and horizontal centrifuge supernatants titred 0 03 cc of 10^{-7} dilution and were non-virulent after 20 minutes' irradiation The results of the immunity test are shown in Table IX

This experiment shows that 1 per cent brain virus supernatants, following centrifugation at 500 or 3,000 R P M, remain equally virulent, that they may be exposed to ultraviolet light, and rendered avirulent in 20 minutes, and finally that they immunize mice in 1 cc doses against at least 10,000 intracerebral lethal doses of street virus

Experiment 11 —This experiment was run in conjunction with Experiment 8 The portion of 1 per cent virus set aside for treatment with chloroform received chloroform to make a 1 per cent concentration The suspension was then shaken for 2 minutes in a mechanical shaker, and for 2 minutes daily thereafter The material proved virulent after 6 days when injected intracerebrally into mice but not after 11 days

Batches of mice were injected with 0 1 cc and 0 1 cc of a 0 1 per cent suspension of

TABLE IX

Immunization of Mice with Graded Doses of 1 Per Cent Irradiated Mouse Brain Rabies Virus against Intracerebral Test Infection

Supernatant—horizontal centrifuge
Virulence before irradiation (0 03 cc in dilutions intracerebrally) 10^{-5*} – 4/4 10^{-6} – 4/4 10^{-7} – 3/4 10^{-8} – 0/4
following (0 03 cc undiluted) 20 minutes – 0/5
Supernatant—Swedish centrifuge
Virulence before irradiation (0 03 cc in dilutions intracerebrally) 10^{-5} – 4/4 10^{-6} – 3/4 10^{-7} – 2/3 10^{-8} – 0/4
following (0 03 cc undiluted) 20 minutes – 0/5

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intracerebrally (0 03 cc) in dilutions					Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		
No vaccine	—	3/3	4/4	1/4	3/4	10^{-5}+	
1 per cent 0.25 cc., 4 doses (3 300 000 M.D ‡)							
"Horizontal supernatant	0/4	0/4	0/4	—	—	$<10^{-1}$	10 000+
Swedish '	0/4	0/4	0/4	0/2	—	$<10^{-}$	10 000+

Footnotes the same as in Table I

TABLE X

Immunization of Mice with Graded Doses of Irradiated and Chloroformized Mouse Brain Rabies Virus

Virulence before irradiation (0 03 cc 10^{-7} dilution intracerebrally)
following (0 03 cc undiluted) 35 minutes—0/6

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions							Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	1/10	1/40	1/80	1/160	1/320	1/640	1/1,280		
Group 1 No vaccine	—	—	4/6	4/6	2/6	1/6	2/6	250	
2 0 5 cc. irradiated (1 650 000 M D ‡)	0/6	0/6	0/6	0/6	0/6	0/6	—	<10	25+
3 0 1 cc irradiated (330 000 M.D)	0/6	0/6	0/6	0/6	0/6	0/6	—	<10	25+
4 0 5 cc chloroformized (1 650 000 M.D)	0/6	0/6	0/6	0/6	0/6	0/6	—	<10	25+
5 0 1 cc chloroformized (330 000 M.D)	1/6	0/6	0/6	0/6	0/6	0/6	—	<10	25+

Footnotes the same as in Table I

the vaccine respectively 3 weeks later these mice together with those given the irradiated virus and the controls, received the test virus intramuscularly in twofold dilution

The results of the test are shown in Table VII The mice given chloroformized vaccine withstood 26 and 16 5 times as much virus as non-vaccinated mice but only one-third as much as the mice given equivalent doses of irradiated vaccine respectively

Experiment 12 —A 1 per cent mouse brain virus was prepared as in Experiments 7 and 9 One portion was irradiated 35 minutes and proved non-virulent by mouse inoculation, a second portion was treated with 1 per cent chloroform and proved non-virulent after 15 days 48 days later, batches of mice received a single intraperitoneal injection of 0 5 or 0 1 cc of the irradiated or chloroformized vaccine respectively 6 weeks later, all mice, together with unvaccinated controls, were given a test intramuscular injection of virulent virus in a dose of 0 01 cc in graded twofold dilutions

The results are shown in Table X Test virus in the non-vaccinated mice showed a titration end point of 0 01 cc of 1 to 250 dilution, as contrasted with less than 1 to 10 in all vaccinated mice The twenty-fivefold difference indicates considerable immunity in both irradiated and chloroformized vaccine groups

Relative Immunizing Potency for Mice of Irradiated Mouse and Dog Brain Vaccines

The next step in developing a vaccine was to compare the titres of virus in infected brains of young mice with those in young guinea pigs, rabbits, and dogs Repeated tests showed that dogs alone, injected with virus intracerebrally when 1 month old, yielded brain tissue with virus titres equal to those in infected mouse brains, namely, 0 03 cc of the 10^{-6} or 10^{-7} dilution Vaccines were prepared with infected dog brains and results obtained which paralleled those with mouse brains

Experiment 13 —An infected dog brain weighing 64 gm was homogenized in a mechanical shaker, diluted with buffer to form a 1 per cent emulsion, titrated for virulence, spun in a horizontal centrifuge at 500 R P M for 5 minutes, distributed in 35 cc quantities in quartz flasks, irradiated 20 minutes with ultraviolet light, tested again for virulence, and stored in the ice box 3 weeks later, groups of mice were vaccinated as follows Group 1 received 0 2 cc intraperitoneally and group 2 received 0 2 cc of the 1 per cent vaccine diluted ten times A third group received 0 2 cc of a commercial chloroformized vaccine, a fourth 0.2 cc of the same vaccine diluted ten times, and a final group was left unvaccinated as controls 3 weeks later the immunity of the mice was tested by giving them 0 01 cc. of street virus intramuscularly in graded doses

The 1 per cent centrifuged supernatant titred 0 03 cc of the 10^{-7} dilution and, following irradiation, failed to kill mice Results of the immunity test are shown in Table XI The titre of street virus in the unvaccinated was approximately 0 01 cc of the 1 to 160 dilution, whereas that in mice

TABLE XI

Immunisation of Mice with Graded Doses of Irradiated and Chloroformed Dog Brain Rabies Virus

Virulence before irradiation (0 03 cc. 10^{-7} dilution intracerebrally) following (0 03 cc undiluted) 20 minutes—0/6

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions			Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	1/10	1/40	1/160		
Group 1 1 per cent 0.2 cc. irradiated	1/9	0/8	0/8	<10	16+
" 2 0 1 per cent, 0.2 cc. irradiated	5/9	4/8	1/8	40	4
3 20 per cent, 0 2 cc chloroformized	2/8	0/8	0/8	<10	16+
4 2 per cent, 0 2 cc. chloroformized	2/8	2/8	1/8	10	16
5 No vaccine	7/9	5/8	3/8	<160	

Footnotes the same as in Table I

TABLE XII

Immunisation of Mice with Graded Doses of Irradiated Dog Brain Rabies Virus after 9 Months Storage

Virulence before irradiation (0 03 cc 10^{-7} dilution intracerebrally) following (0 03 cc undiluted intracerebrally) 30 minutes—0/6

Immunity of Vaccinated Mice

Dose of vaccine	Mortality of mice given test virus intracerebrally (0.03 cc) in dilutions							Titre† of virus in mice	Difference in titre of virus in vaccinated and non vaccinated mice
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
Group 1 No vaccine	—	—	4/4	4/4	4/4	1/4	0/4	10^{-6}	
2 1 per cent 0 25 cc. 4 doses irradiated 15–30 min	—	1/4	1/4	1/4	0/4	—	—	10^{-4}	100
" 3 1 per cent same dose irradiated 90–120 min	0/2	1/4	0/4	0/4	0/4	—	—	$<10^{-2}$	10 000+
4 6 6 per cent same dose chloroformized	—	2/3	2/4	3/4	1/4	—	—	10^{-6}	10

	Mortality of mice given test virus intramuscularly (0 01 cc) in dilutions						
	1/10	1/40	1/160	1/640	1/2,560		
Group 5 No vaccine	3/3	4/4	1/4	1/4	2/4	640	
" 6 1 per cent 0 2 cc irradiated 15–30 min.	0/4	1/4	0/4	0/4	0/4	<10	64+
7 1 per cent, same dose irradiated 90–120 min	2/4	0/4	0/4	0/4	0/4	10	64
8 6 6 per cent, same dose chloroformized	3/4	1/4	1/4	0/4	1/4	35	18

Footnotes the same as in Table I.

receiving the 1 per cent irradiated or 20 per cent chloroformized vaccines was at least sixteen times, and in mice receiving 0 1 per cent irradiated or 2 per cent chloroformized vaccine, four to sixteen times greater, demonstrating considerable immunizing potency of both the 1 per cent irradiated dog brain and the 20 per cent chloroformized preparations

A final experiment is submitted to show the effectiveness of the 1 per cent irradiated dog brain virus after 9 months at ice box temperature

Experiment 14 —A 1 per cent dog brain virus was prepared and tested for virulence according to the method described in the previous experiment Portions were exposed to ultraviolet light for 15 and 30 minutes, and for 90 and 120 minutes Samples were tested for virulence at varying intervals from 3 to 30 minutes The original material titred 0 03 cc $\times 10^{-7}$ and at 30 minutes virus failed to kill mice The various samples were then stored in the ice box at 40°F

9 months later, mice were treated as follows Group 1 remained unvaccinated Other groups of mice were vaccinated intraperitoneally in the following manner Group 2 was given 0 25 cc of the pooled 15 and 30 minute vaccines every other day four times, group 3 received the same dose of pooled 90 and 120 minute vaccines, group 4 was given the same dose of a commercial chloroformized vaccine diluted five times, group 5 was left as a second batch of controls, group 6 was given a single dose of 0 2 cc of the 15 minute irradiated vaccine, group 7 a single dose of 0 2 cc of the 120 minute irradiated vaccine, and group 8 the same dose of the chloroformized vaccine diluted 1 to 5 3 weeks later the first four groups were tested for immunity to an intracerebral injection and groups 5 to 8 were tested for immunity to an intramuscular injection of virulent virus

Table XII shows that a total of 1 cc of 1 per cent irradiated dog brain vaccine immunized mice against 100 to 10,000 intracerebral lethal doses and at least 64 intramuscular doses The chloroformized vaccine immunized mice against 10 intracerebral test doses and 18 intramuscular doses

DISCUSSION

From a practical viewpoint, culture virus has not yet proved a satisfactory source of rabies vaccine, due chiefly to its low content of virus To immunize, approximately 1 cc is required for mice and 500 cc for dogs,—about 5 per cent of the body weight

The supernatant of centrifuged brain tissue virus, however, has proved a good source of vaccine The virus content of infected brain tissue per unit volume is 1,000 times that of tissue culture A 10 per cent emulsion can be centrifuged to sediment a large portion of the tissue fragments without lowering the titre of virus The supernatant from a 1 to 5 per cent emulsion can be irradiated so as to destroy virulence without loss of immunizing potency 0 1 cc of such a preparation immunizes mice adequately,—0 5 per cent of the body weight

Altogether the results of these experiments to date suggest that basically the immunizing potency of a vaccine is dependent upon virus content, that is, that the immunizing antigen is the virus particle They indicate also that one intracerebral lethal mouse dose of a given strain of virus from tissue culture is equivalent in immunizing potency to one dose of the same strain from infected mammalian brain Finally, the findings point to a relation between number of mouse lethal doses required to immunize and body weight

The 1 per cent irradiated dog brain virus has proved an effective and practical vaccine for immunizing mice, and equal or superior to chloroformized vaccine It is now being tested in dogs with promising results (4)

SUMMARY

In the experiments described above, we found with respect to tissue culture rabies virus that 1 cc , which contains approximately 50,000 mouse intracerebral lethal doses, properly irradiated, was required to immunize a mouse, 500 cc , which contain 25,000,000 doses, were required to immunize a 20 pound beagle dog

Tissue culture virus concentrated ten times proved capable of immunizing mice in a dose one tenth as large as that required for unconcentrated culture virus

Brain virus suspensions were centrifuged so as to remove a large part of the tissue particles without striking loss in the virulence of the supernatant The centrifuged supernatants of 1 to 5 per cent brain virus suspensions were irradiated so as to destroy virulence and yet retain immunizing potency

Irradiated supernatants of mouse brain virus proved capable of immunizing mice as well as or better than similar supernatants treated with chloroform

0 1 cc of a 1 per cent irradiated dog brain virus containing approximately 50,000 mouse intracerebral lethal doses immunized mice effectively

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ORGAN SPECIFICITY OF TISSUES OF THE DOG AND MAN AS SHOWN BY PASSIVE ANAPHYLAXIS IN GUINEA PIGS*

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In previous studies (1, 2) it was shown that prolonged immunization of rabbits with sedimented bacterial vaccines grown in infusion broths prepared from the tissues of different animals, stimulated the production of antibodies not only for the bacteria but also for the broths It was demonstrated that fatal anaphylaxis usually followed the injection of homologous organ broths into guinea pigs passively sensitized with the antisera of rabbits immunized in this way Thus, almost complete immunological specificity for broths made from striated muscle and brain was so obtained It was found that some of the organ specific substances still remained in infusions of these tissues after they had been autoclaved The partial destruction or conjugation of the relatively small amounts of blood in the infusions by such treatment, seemed to provide a simple method which gave promise of possible application to various tissues in an effort to demonstrate organ specificity by the elimination of the numerous cross reactions which are usually obtained in immunological tests with suspensions and extracts of unheated cells

Reviews of the voluminous literature on recent studies of organ specificity carried out by various methods, have been made by Witebsky (3), Landsteiner (4) and others, to which the reader is referred. It should be stated, however that most of these investigations have been made by means of either the complement fixation or the precipitin reaction with alcoholic extracts of fresh organs Sometimes aqueous solutions or suspensions of fresh tissues were used as antigens (5) In a few instances particular protein fractions have been tested By serological methods more or less strict organ specificity has been shown for brain (6 7), testicle (7), kidney (8–10), suprarenal gland (11) placenta (12, 13) epiphysis (14) hypophysis (15), stomach and intestine (16) lens and intestine (17) leucocytes (18), carcinoma and sarcoma (19–22) and glioma (23) With some organs the specificity of antisera has been shown *in vivo* either directly by the action of cytotoxins as in the case of the injection of anti kidney sera for the production of nephritis (24, 25) or indirectly by the effects on metabolism induced by the injection of antisera for thyroid gland and the anterior body of the pituitary gland (26)

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As to anaphylaxis, it has supplied a delicate technique for investigations of organ specificity (27) Anaphylaxis can be observed directly when a very small amount of a specific antigen is injected into a sensitized guinea pig, and indirectly desensitization can be noted with a much smaller amount of the specific substance Active anaphylaxis was used by Pfeiffer (28) for the study of the organ specific properties of the proteins of the liver, spleen, kidney, blood, etc The results seemed to indicate the existence of such specificity, although Ranzi (29) had previously obtained entirely negative results, and Pearce, Karsner, and Eisenbrey (30) failed to confirm Pfeiffer's claims However, Kraus, Doerr, and Sohma (31) found that guinea pigs which had been sensitized to the protein of the crystalline lens of one species would react to lens protein in general The same thing was demonstrated for testicular protein by von Dungern and Hirschfeld (32) The results in this case, as shown by the localized reaction of Arthus, seemed to be less specific than in the case of lens

Because of the delicacy of the reaction of anaphylaxis and the numerous species-specific cross-reactions which have been obtained with most anti-organ sera when aqueous extracts of fresh tissues were used as antigens, the method has long been neglected as a technique for the demonstration of organ specificity Our own investigations on passive anaphylaxis with broths, already referred to, seemed to open a new approach to the subject

EXPERIMENTAL

In view of the fact that the serological tests carried out by a number of investigators, had shown more or less specificity for different organs, not only with lipoids but also with whole cells and protein fractions, it occurred to us to determine whether such specificity could be generally demonstrated by anaphylactic tests with the water-soluble hydrolytic products of various autoclaved tissues, that is, with broths, according to the method previously used successfully with broths prepared from striated muscle (1) and brain (2) To this end, guinea pigs were passively sensitized with antisera from rabbits to determine (*a*) tissue specificity of different organs of the dog and man, (*b*) differences in the organ-specific properties of corresponding tissues in these two species, (*c*) relationship of the Forssman antigen to organ specificity, (*d*) tissue specificity of human fibromyoma and melanotic sarcoma, (*e*) relationship of the vascularity of different organs to their cross-reactions with antisera for whole blood broths The passively sensitized guinea pigs were injected with autoclaved extracts of various organs and, if the animals survived, they were injected one hour later with the homologous extract to determine possible antigenic relationships by specific desensitization

In the present study, two kinds of fluids have been used As antigens for the production of tissue-specific antisera, *Pasteurella boviseptica* was

grown in certain autoclaved organ extracts to which peptone, glucose, and sodium chloride had been added The sedimented heat killed bacteria only, to which some of the particular broth was probably adsorbed, were injected into rabbits Broth alone in large amounts was practically non antigenic We have found (1) that a number of other bacteria, as well as animal charcoal, kaolin, or collodion particles can be substituted for *Pasteurella bovisepticа* when grown in ordinary beef infusion broth or added to it, and then centrifuged, heated, and used as vaccines The antigens for anaphylactic tests in guinea pigs were autoclaved organ extracts to which sodium chloride alone was added

Methods

Broths for Growth of Bacterial Vaccines —The organs of normal dogs which had been sacrificed by operative ether anesthesia, were removed, dissected as free as possible from fat and connective tissue cut into small pieces washed under running tap water, put through a meat grinder, washed over several layers of gauze with physiological salt solution, and one part of tissue mixed with two parts by weight of distilled water After standing in the ice box for 48 hours the infusions were autoclaved at 122–124 C for 20 minutes filtered through gauze and then through paper and 0 5 per cent by weight of sodium chloride added to them The filtrates were adjusted to pH 7.2–7 4 with the idea of causing as much destruction of any common blood antigen as possible by the subsequent heating without complete destruction of the predominating fixed tissue antigens The extracts were steamed in the Arnold sterilizer for 15 minutes filtered through paper put into 200 cc Erlenmeyer flasks, and sterilized in the autoclave at 122–124 C for 20 minutes The final pH of the extracts varied from 7 0 to 7 2 The extracts of human organs were prepared in the same manner The tissues were collected from six different individuals at autopsy and at operations None of the materials were obtained from persons with acute infections

Bacterial Vaccines —After the addition of sterile Difco peptone (pH 7 6) and glucose to 1 and 0 5 per cent respectively, 100 cc. amounts of the various organ broths were inoculated with 0 01 cc. of a 24 hour beef infusion broth culture of *Pasteurella bovisepticа* and incubated at 37 5 C for 48 hours when heavy growths were obtained The cultures were centrifuged the supernatants decanted and discarded each packed bacterial sediment suspended in 10 cc of 0 85 per cent solution of sodium chloride, and heated at 56 C for one hour

Immunization of Rabbits —Rabbits of mixed American Blue and Dutch stock were immunized by two or three weekly injections of increasing doses of 0 05 to 0 5 cc. of the concentrated bacterial vaccines just described over periods of 6 to 8 weeks until a total of 7 5 to 10 cc had been given Each animal was bled 50 cc. from the ear veins on the 8th day and also on the 10th day after the last injection the serum collected pooled merthiolate added to 1 to 10 000 and stored in the refrigerator until needed for passive sensitization of guinea pigs to the various organ broths Such antisera had been shown to contain antibodies not only for the bacteria but also for the broths

Antigens for Anaphylactic Tests —The antigens used for anaphylactic tests were

either from the same lots of autoclaved extracts of organs used for the growth of the bacterial vaccines described above or extracts of other tissues prepared in exactly the same way Peptone and glucose were not added to these antigens Extracts which showed turbidity were centrifuged to clear them as much as possible of any gross suspended

TABLE I

Specificity of Antisera for Broths Made from Different Organs of the Dog as Shown by Passive Anaphylactic Tests with Guinea Pigs Injected with Autoclaved Aqueous Extracts of Various Tissues of the Dog

Dog organ broth tested for production of shock	Anti broth sensitizing sera and reactions* of guinea pigs to (1) heterologous dog organ broth and (2) homologous dog organ broth											
	Anti skeletal muscle		Anti heart		Anti ileum		Anti lung		Anti kidney		Anti blood	
	1	2	1	2	1	2	1	2	1	2	1	2
Skeletal muscle		4+	4+		−	4+	−	2+	±	±	−	±
Heart	4+			4+	−	4+	−	+	±	−	+	−
Diaphragm	4+		4+		−	4+	−	2+	±	−	−	+
Tongue	4+		4+		−	4+	−	2+	−	−	−	±
Esophagus	4+		4+		±	3+	+	−	±	−	−	+
Stomach	−	4+	−	4+	−	4+	−	2+	−	±	−	3+
Ileum	−	4+	+	2+		4+	3+	−	−	−	±	2+
Colon, cecum	−	4+	−	4+	±	2+	+	−	−	−	±	+
Bladder	−	4+	−	4+	+	±	4+		−	−	−	2+
Aorta	−	4+	−	4+	−	4+	±	−	−	−	±	3+
Lung	±	4+	±	4+	+	+		3+	±	−	±	−
Trachea	−	4+	−	4+	±	4+	+	−	−	−	−	2+
Liver	±	−	±	4+	−	4+	−	−	±	−	3+	−
Kidney	−	4+	±	3+	−	4+	−	±		4+	4+	
Pancreas	−	4+	−	4+	−	4+	−	2+	−	±	−	3+
Spleen	−	4+	±	4+	−	3+	−	2+	±	±	+	−
Omentum, fat	−	4+	−	4+	−	4+	−	3+	−	±	−	2+
Lymph gland	−	4+	−	4+	−	4+	±	+	−	−	±	2+
Parotid gland	−	4+	−	4+	−	4+	−	2+	−	±	−	3+
Thyroid gland	±	4+	−	4+	−	4+	−	2+	−	−	−	±
Testicle	−	4+	−	4+	−	4+	−	3+	±	−	−	3+
Brain	−	4+	±	4+	−	4+	−	3+	−	±	3+	−
Skin	−	4+	−	4+	−	3+	−	+	−	±	−	−
Blood (whole)	−	4+	±	4+	−	4+	±	2+	±	−		4+

* 4+ = fatal reaction with death in 2½ to 5 minutes, 3+ = severe, almost fatal, reaction, 2+ = moderate reaction, + = slight but definite reaction, ± = very slight or doubtful reaction, − = no reaction The signs, symptoms, and autopsy findings were all typical of anaphylactic shock.

matter Because of the high content of epinephrin, extracts of suprarenal glands were primarily toxic by intravenous injection into guinea pigs and were not used in anaphylactic tests None of the other organ extracts were primarily toxic for guinea pigs

Anaphylactic Tests—Guinea pigs of 250 to 300 gm in weight were prepared for anaphylactic tests by intraabdominal injection of 2 cc of the antisera for various broths previously described, and tested for passive sensitization after 48 hours by injection into

the saphenous vein with 2 cc of autoclaved extracts of different tissues of the dog and man In case the guinea pig lived, this injection was followed one hour later by injection into the same vein or opposite vein with 1 5 cc of autoclaved extract of the homologous organ to test for possible antigenic relationships which might be shown by desensitization

Organ Specificity of Antisera for Broths Made from Different Tissues of the Dog

In order to test the specificity of antisera for broths prepared from different tissues of the dog, rabbits were immunized as previously described with sedimented bacterial vaccines grown in broths made from skeletal muscle, heart, small intestine (ileum), lung, kidney, and whole blood The results with autoclaved extracts of various tissues as antigens in anaphylactic tests in guinea pigs passively sensitized with the different antisera, are summarized in Table I Six antisera and twenty four extracts were used It will be seen that the antiserum for skeletal muscle showed practically complete specificity for striated muscle as demonstrated by fatal reactions with guinea pigs injected with extracts of skeletal muscle, heart, diaphragm, tongue, and esophagus, while very slight or negative reactions were obtained with extracts of other tissues The antiserum for heart muscle gave the same reactions as that for skeletal muscle with the exception of a definite cross reaction with ileum This reaction could not have been due to certain amounts of smooth muscle common to both the heart and ileum, because no reactions were obtained with heart antiserum and extracts of other tissues containing large amounts of smooth muscle The antiserum for ileum reacted best with the homologous antigen, but definite anaphylaxis also occurred in the guinea pigs injected with extracts of bladder and lung Smooth muscle is common to these three organs, but it is also present in other tissues, extracts of which gave completely negative reactions It is possible that the presence of epithelial cells might account for the more or less definite cross reactions of esophagus, colon, bladder, lung, and trachea with the antiserum for ileum This is further indicated by the reactions of the antiserum for lung with extracts of esophagus, ileum, colon, bladder, and trachea Furthermore, in those instances in which the reactions were not fatal, there was complete desensitization to later injections of the homologous extract of lung It should be mentioned, however, that these tests were complicated by the fact that the antiserum for dog lung contained Forssman antibodies The hemolytic titer for sheep red cells was about 2500 and the serum was primarily toxic for guinea pigs, causing death in 3 minutes when as small an amount as 0 2 cc was injected intravenously When injected intraabdominally with 2 cc of the serum, about 50 per cent of the animals died All survived when given 1 cc by this route and these

guinea pigs were used in the passive anaphylactic tests with this antiserum The antisera for other dog organs did not seem to be primarily toxic when injected intraabdominally in doses of 2 cc , although all of these antisera showed hemolytic titers somewhat higher than normal for sheep red cells It is possible that the toxicity of the antiserum for dog lung was due not only to Forssman antibodies but also to organ-specific antibodies for lung itself

In addition to the almost identical properties of the antisera for skeletal muscle and heart which have already been referred to, the special characteristics of antisera for kidney and whole blood deserve attention The antiserum for kidney gave very slight reactions with a considerable number of heterologous organ extracts, but it gave a fatal reaction only with the extract of kidney itself This antiserum was peculiar, however, in that either complete or almost complete desensitization occurred following the injections of the passively sensitized guinea pigs with any of the extracts of various heterologous tissues Theoretically, an antiserum for such an organ would have a high degree of polyvalency, although the antibody response to any one of the complex mixture of haptens might be very slight, but sufficient to cause more or less reaction to a corresponding antigen and thus desensitization in anaphylaxis In this connection, another antiserum which showed an even greater degree of apparent polyvalency which can be more easily explained, was that for whole blood Guinea pigs passively sensitized with this antiserum had fatal reactions when injected with extracts of whole blood and kidney, severe reactions with those from liver and brain, and definite reactions with extracts of heart and spleen All these organs are especially well supplied with blood and, since in the preparation of the various antigens no attempts were made to wash all of the blood out of any of the tissues, some of them probably contained a good deal of it It is not surprising then that marked cross-reactions were obtained with the autoclaved extracts of some of the tissues mentioned above, when tested against the antiserum for blood It is surprising, however, that a larger number of cross-reactions were not obtained with this antiserum

Organ Specificity of Antisera for Different Human Organ Broths

Since we had been able to demonstrate almost complete tissue specificity with antisera for a number of different organs of the dog in passive anaphylaxis by the methods described above, we were interested to determine if the same technique could be applied equally well to show immunological specificity of human tissues If such proved to be the case, it was hoped that it would then be possible to compare the anaphylactogenic properties

of normal organs and different kinds of tumors of man and animals Accordingly, antisera for autoclaved aqueous extracts of seven representative human tissues were produced by the methods described in a previous section of this paper The normal tissues used were skeletal muscle, lung, liver, kidney, blood, and placenta Two tumors were available These

TABLE II

Specificity of Antisera for Broths Made from Different Human Tissues as Shown by Passive Anaphylactic Tests with Guinea Pigs Injected with Autoclaved Aqueous Extracts of Various Human Tissues

Human organ broth tested for production of shock	Anti-broth sensitizing sera and reactions of guinea pigs to (1) heterologous human organ broth and (2) homologous human organ broth													
	Anti skeletal muscle		Anti lung		Anti liver		Anti kidney		Anti placenta		Anti fibromyoma		Anti-blood	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Skeletal muscle		3+	−	3+	−	4+	−	3+	−	3+	−	+	−	4+
Diaphragm	3+	−	−	3+	−	4+	−	3+	−	4+	−	+	−	4+
Heart	4+		−	3+	−	4+	−	2+	−	3+	−	±	−	4+
Esophagus	2+	−	−	3+	−	4+	−	3+	−	3+	−	+	−	4+
Ileum	−	3+	+	−	−	4+	−	4+	−	4+	−	2+	−	4+
Uterus	−	2+	+	±	4+		±	2+	+	−	−	±	4+	
Fibromyoma	−	3+	−	4+	±	4+	−	3+	−	4+		+	±	4+
Placenta	−	2+	±	3+	±	−	−	4+		4+	−	+	4+	
Aorta	−	3+	±	2+	±	−	−	3+	−	3+	−	±	±	4+
Lung	−	2+		4+	4+		±	2+	−	3+	−	±	4+	
Trachea	−	3+	+	−	+	−	−	3+	−	3+	−	+	±	3+
Liver	−	3+	+	−		4+	±	3+	±	2+	−	2+	4+	
Kidney	−	2+	±	3+	+	−		3+	−	3+	−	±	+	+
Spleen	−	2+	−	3+	+	±	±	2+	±	2+	−	+	4+	
Lymph gland	−	3+	−	2+	−	4+	−	3+	−	3+	−	+	−	4+
Melanotic sarcoma	−	3+	−	2+	−	4+	−	3+	−	3+	−	±	−	4+
Skin	−	2+	−	4+	−	4+	−	3+	−	2+	−	+	−	4+
Fat	−	2+	−	3+	−	4+	−	2+	−	2+	−	±	−	4+
Brain (ox)	−	3+	−	4+	−	4+	−	3+	−	3+	−	+	+	2+
Blood (whole)	−	3+	−	3+	−	4+	−	2+	+	−	−	+		4+

The signs indicating the degree of shock are to be interpreted as in Table I

were fibromyoma of the uterus and melanotic sarcoma, the latter from two different individuals Seven different antisera were tested with twenty autoclaved extracts The results are given in Table II As in the case of the tests with antisera for various organs of the dog, the only completely and definitely specific antiserum was that for skeletal muscle This serum reacted only with extracts made from organs containing striated muscle The antiserum for fibromyoma reacted only with the extract of this tumor but this serum was of low potency and the reactions were slight The ex-

tracts made from fibromyoma and uterine muscle had a jelly-like consistency when first brought from the ice box and became completely liquefied only when warmed to about 25°C They appeared to contain a considerable amount of gelatin, but they did not cause colloid shock and were not primarily toxic when injected intravenously into normal guinea pigs Studies carried out by Bailey and Gardner (unpublished data) since the completion of these experiments, indicate that a more potent antiserum could have been obtained against the extract of fibromyoma if the tissue infusion had been adjusted on the acid side of neutrality, at pH 6 7–6 8, before each autoclaving An alkaline reaction of about pH 7 2 was used in the present experiments

Two other anti-human sera which appeared to be almost completely tissue-specific were those for kidney and placenta The antiserum for the former reacted very slightly with four different antigens, but there was no desensitization The antiserum for placenta gave a slight but definite reaction with the extract of uterus This cannot be explained on the basis of antibodies for uterine decidua since the uterine antigen was made entirely from smooth muscle The antisera for blood, liver, and lung also reacted with the extract of uterus It does not seem probable that these reactions were due to the antigen of blood, because the uterine muscle used was not very vascular and could have had only a small amount of blood in it Furthermore, antisera for the highly vascular tissues of lung and liver gave no reactions with the extract of whole blood itself It will be noted, however, that the antiserum for lung gave a definite reaction with extract of liver, and the antiserum for liver produced a fatal reaction with extract of lung

The antiserum for human whole blood gave fatal anaphylactic reactions with the homologous antigen as well as with extracts of uterus, placenta, lung, liver, and spleen It even reacted slightly with an antigen made from ox brain in which the blood was heterologous We have already mentioned that the antiserum for dog blood produced a severe reaction with an extract of dog brain It is probable, however, that the cross-reactions of certain tissue extracts with antisera for whole blood, were due to the relatively large amounts of blood still present in such highly vascular organs as the lungs, liver, spleen, etc , from which the heated antigens were made Extracts of tissues which normally seemed to contain only small amounts of blood, gave little or no reaction with the antiserum for whole blood

DISCUSSION

The methods of production of immune sera and carrying out anaphylactic tests used in the present investigation proved to be very well adapted for

the demonstration of organ specificity By the use of autoclaved extracts of aqueous infusions of various tissues, the numerous cross reactions commonly observed in ordinary serological tests with unheated suspensions of entire organs or with alcoholic extracts of different tissues, were either entirely eliminated or greatly reduced in number The proteins of the tissue infusions were subjected to hydrolysis by steam under pressure Our work is somewhat similar to that of Fink (33) who studied the proteose fractions obtained by hydrolysis of egg albumin in the autoclave This investigator found some evidence of very slight antigenic activity by means of complement fixation, precipitin, and anaphylactic tests with certain of the hydrolytic fractions of egg white The fractions were injected into rabbits and guinea pigs, but were not combined with a bacterial vaccine to serve as a carrier of the tissue antigen or hapten as was done in the present experiments to increase the immunizing power of the hydrolytic products

The results of the present study are interesting because of the relatively high degree of tissue specificity which can be obtained with heated antigens It would seem that the subjection of aqueous extracts of organs to steam under pressure might increase their immunological specificity in two ways Zoet (34) has reported the production of artificial horse swine "hybrid" protein by autoclaving mixtures of the sera These synthetic or "coupled" proteins were found to possess one or more serological factors not present in horse serum or pig serum In the case of the extracts of tissues used by us, the union of organ specific substances with any blood still present might produce new specific immunological factors not common to blood, the organ itself, or to other organs with different combinations It should be stated in this connection, however, that the findings of Zoet could not be confirmed by Nigg (35) The other way in which autoclaving tissues might increase their specificity would be the destruction of a more thermolabile antigen, leaving some of the greatly predominant organ specific antigen to exert its immunizing or anaphylactic effects when injected into animals This would be similar to boiling sheep red cells to destroy the isophile antigen common to both the sheep and the ox, leaving the thermostable heterophile antigen which, in the case of the sheep, is found almost exclusively in the red blood cells When such boiled red cells are injected into rabbits, highly specific hemolytic sera are produced

In this connection, it is interesting to consider some of the known thermostable antigens which might be distributed throughout the body and, because of their identity or similarity in different organs, give cross reactions in immunological tests and thus obscure the reactions of organ specific substances It is known that the blood contains such thermostable antigens derived from proteins as shown by the use of boiled serum in the

production of species-specific antibodies for blood serum Furthermore, species-specific lipoids are thermostable and antigenic under certain conditions In addition, some lipoid-polysaccharide-protein complexes, such as the Forssman antigen, are highly thermostable The question now arises as to whether this particular antigen might not interfere with the demonstration of organ specificity in the case of some animals in which it may be more or less uniformly distributed in all the tissues The production of a serum containing both organ-specific antibodies and heterophile antibodies, with guinea pig brain as the antigen, has been reported by Witebsky (36) In complement-fixation tests with such an antiserum and alcoholic extracts of various organs containing the Forssman antigen, a certain amount of confusion due to cross-reactions is unavoidable In the present study, passive anaphylactic tests were carried out with the guinea pig which belongs to the Forssman group of animals When the results of injections of aqueous extracts of different organs of the dog, all the tissues of which contain the Forssman antigen, were compared with the results obtained with extracts of human tissues which do not contain this antigen, no definite differences in specificity were apparent It would seem therefore that the particular method of passive anaphylaxis we have employed might be applied successfully for the demonstration of organ specificity with the tissues of various animals, irrespective of the presence or absence of the Forssman antigen or similar ubiquitous substances

SUMMARY

The immunization of rabbits for periods of 6 to 8 weeks with sedimented, heat-killed vaccines of *Pasteurella bovisepticа* grown in infusion broths made from six different tissues of the dog and seven tissues of man, caused the production of sera containing antibodies for the broths as well as for the bacteria The broth made from human fibromyoma of the uterus was the least antigenic of all, as indicated by passive anaphylactic tests in guinea pigs When these animals were prepared by intraabdominal injection with the rabbit antisera and tested 48 hours later by intravenous injection with autoclaved aqueous extracts of a large number of organs of the dog and man, the guinea pigs were found to be passively sensitized so that severe or fatal anaphylaxis was generally obtained with broths made from the homologous organ and in some instances with those prepared from heterologous organs of the same species In most instances, the injection of broths from heterologous tissues did not desensitize to later injections of that from the homologous tissue The most organ-specific antisera were

those for striated muscle, small intestine (ileum), kidney, placenta, and fibromyoma, and the least so those for whole blood, liver, and lung The cross reactions of the antiserum for blood were mostly with extracts of tissues which normally contain large amounts of blood The presence of Forssman antigen in the tissues of the dog did not interfere with the demonstration of organ specificity by the methods used In general, the results indicate that the various tissues of man and the dog contain thermostable, water soluble, organ specific substances which can be demonstrated by passive anaphylaxis in guinea pigs The chemical nature of these substances has not been definitely determined, although there are some indications that they are protein split products, probably proteoses

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The observations of Much (14) and Rosenthal (16, 18) appear of special interest since these workers reported that their strains exhibited lytic activity not only against Gram-positive organisms, but also against Gram-negative species An effort was therefore made to isolate from natural sources strains of aerobic sporulating bacilli possessing the biological activities of the cultures studied by Much and Rosenthal

Isolation of Aerobic Sporulating Bacilli Exhibiting Bactericidal Activity

The material (soil, sewage, manure, cheese, etc) to be investigated for the presence of aerobic, spore-forming antagonists was heated at 70°C for 30 minutes to destroy the non-sporulating forms The heated material was then inoculated into suspensions of living cells of *Escherichia coli* or *Staphylococcus aureus*, these bacterial suspensions, containing approximately 5×10^8 cells per cc , were prepared by resuspending the bacterial cells centrifuged from 8 hour old broth cultures into phosphate buffer (M/15) at pH 7 3 Frequent microscopic and cultural tests were made in an attempt to determine the presence of an antagonistic flora capable of destroying the staphylococci or colon bacilli Cultures exhibiting antagonistic activity were immediately inoculated into new suspensions of living cells of the same test organisms In all cases it was found that the addition of small amounts of peptone or gelatin (0 01 per cent) to the bacterial suspension greatly accelerated the disappearance of the staphylococci or colon bacilli Under optimum conditions, complete disappearance of the staphylococci could often be observed in 18 to 24 hours at 37°C , it usually took 48 to 72 hours to cause the destruction of the Gram-negative bacilli At this stage isolation of the active strain of antagonist was readily obtained by heating the mixed culture at 75°C , and plating it on peptone agar

Many different strains of aerobic sporulating bacilli endowed with properties antagonistic to other microorganisms were isolated by the use of this technique, 7 from soil, 3 from manure, 2 from sewage, and 2 from cheese, all were found active against Gram-positive microorganisms and also, but to a smaller degree, against Gram-negative bacilli It was also possible to obtain from the Culture Collection of the Lister Institute, London, through the courtesy of its curator, Dr St John Brooks, 6 of the *Tyrothrix* cultures isolated by Duclaux in 1887, and tested by Rosenthal As described by the latter worker, the *Tyrothrix* cultures—especially *Tyrothrix scaber*—were found to exhibit bactericidal properties, although they appear much less active than the cultures isolated in the present work Finally, it was found that *Bacillus brevis* (strain B G) from which gramicidin was first isolated, can also cause the destruction of Gram-negative bacilli resuspended in very dilute (0 003 per cent) peptone solutions

A complete descriptive study of these different strains of aerobic sporulating bacilli has not been carried out, it can be stated, however, that they appear to belong to different bacterial species since they differ in many

morphological, cultural, and physiological characteristics such as staining reactions, morphology, colony appearance, manner of growth in broth, inhibitory effect of glucose, thermophilic properties, liquefaction of starch, production of bactericidal substances, etc

Separation of a Soluble Bactericidal Fraction by Extraction of the Cultures with Ethyl Alcohol

It has been shown elsewhere (5) that the bactericidal principles produced by *Bacillus brevis* can be obtained in solution by extracting the cells or peptone cultures of this organism with ethyl alcohol or acetone at acid reaction The following experiments describe the procedures used to prepare alcoholic solutions possessing bactericidal activity, from cultures of several of the organisms mentioned in the preceding chapter

The cultures were grown in two different media (*a*) 1 per cent tryptone 0 5 per cent NaCl tap water—pH 7 0, and (*b*) 1 per cent gelatin 0 05 per cent $MgSO_4$ 0 2 per cent KH_2PO_4 0 4 per cent Na_2HPO_4 0 5 per cent NaCl tap water—pH 7 0[2] The media were distributed in shallow layers (2 cm thick) and autoclaved at 15 pounds pressure for 30 minutes They were inoculated with peptone cultures of the selected organism previously heated at 75 C , 0 5 cc inoculum was used per liter of medium Incubation was allowed to proceed for 6 days at 37°C

At the end of the incubation period the cultures were adjusted to pH 4 7 with concentrated HCl this required 3 5 to 4 5 cc of acid per liter of culture The acidified cultures were allowed to stand for 24 hours at room temperature, they were then centrifuged and the supernatant fluid discarded The precipitates were taken up in 95 per cent alcohol, using 50 cc of this solvent per liter of original culture On the following day the alcoholic solutions were clarified by filtration through filter paper they were then diluted with 10 volumes of 1 per cent solution NaCl in tap water A precipitate formed which contained the active principle it was separated by filtration and desiccated over P_2O_5 *in vacuo* The yield of precipitate varied markedly from one culture to another the largest yields were recovered from cultures of *Bacillus brevis* (strain B G), of culture T C[3] (isolated from a Turkish cheese) and of culture LBa[3] (obtained from sewage) Up to 500 mg of dry material was recovered from 1 liter of culture of these organisms

The dried material was dissolved in 95 per cent alcohol to give solutions containing 20 mg per cc The alcoholic solutions, diluted in distilled water give opalescent colloidal solutions which precipitate on addition of electro

[2] When purified gelatin was used, growth was much stimulated by the addition to the medium of small amounts of yeast extract or meat infusion which probably supplied some accessory growth factors

[3] Cultures T C and LBa were isolated at the laboratory of the Hospital for Incipient Tuberculosis Ray Brook New York, in cooperation with Dr D Yagin and Mr L Baisden

exhibits bactericidal activity against both test organisms, resuspended in buffer solutions This correlation between the reaction of the cell to the Gram stain and its differential susceptibility to gramicidin has been extended to a number of other bacterial species, pneumococci, streptococci, staphylococci, diphtheria and diphtheroid bacilli, aerobic and anaerobic sporulating Gram-positive bacilli, have all been found to be susceptible to both gramicidin and tyrocidine On the contrary, the following Gram-negative groups, *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Hemophilus*, *Neisseria*, are resistant to gramicidin but susceptible to tyrocidine

Another generalization appears justified at the present time Treatment with tyrocidine often results in the lysis of the bacterial cells (streptococci, diphtheria and diphtheroid bacilli are an exception to this rule) On the contrary, when the cells of susceptible bacterial species are treated with gramicidin, they retain their characteristic morphology and staining reactions long after they have lost the capacity to grow when inoculated into plain broth or on plain agar

It is also apparent from the results presented in Table I that both gramicidin and tyrocidine are more effective when tested in buffer solutions than in the presence of the constituents of meat infusion peptone broth In particular, the activity of tyrocidine against *E coli* (and other Gram-negative bacilli) is remarkably inhibited when the bacterial cells are resuspended in peptone solutions or meat infusion peptone broth

The Activity of Gramicidin and Tyrocidine against Bacterial Infections

As reported elsewhere (9, 10), the intraperitoneal injection of gramicidin exerts a protective action against infection of mice with pneumococci and streptococci, gramicidin is ineffective *in vitro* against Gram-negative bacilli and also fails to protect mice against infection with *Klebsiella pneumoniae* Tyrocidine, on the contrary, can be shown to exert a bactericidal effect *in vitro* against Gram-negative as well as Gram-positive microorganisms, however, all attempts to obtain a protective effect with this substance against Gram-negative infections have so far failed

Varying amounts of tyrocidine have been administered to mice by the intraperitoneal, subcutaneous, intravenous, or oral routes, and failed to protect these animals against infection with *Klebsiella pneumoniae* or *Salmonella aertrycke* In fact, the feeding of large amounts of tyrocidine to mice even failed to modify the normal Gram-negative intestinal flora It can be stated in passing that large amounts of young active cultures of aerobic sporulating bacilli (*Bacillus brevis* strain B G, culture T C, and *Tyrothrix scaber*) have been fed to mice and guinea pigs in an attempt to

modify the intestinal flora as suggested by Rosenthal (17), it was indeed possible to recover these bacterial species from the feces for a number of days or even weeks after these cultures had been fed to guinea pigs, showing that the sporulating bacilli had become established in the intestinal tract There was also definite indication that the Gram positive components of the normal intestinal flora had been displaced by the aerobic sporulating bacilli, but in no case could we observe any significant reduction of the number of coliform bacilli

TABLE II

The Protective Effect of Gramicidin and Tyrocidine against Infection of Mice with Type I Pneumococcus*

Treatment (intraperitoneal)		Infecting dose of pneumococci†											
		10^{-4}						10^{-7}			10^{-8}		
	mg												
Gramicidin	0 025	S	S	S	S	S	S	—	—	—	—	—	—
	0 01	S	S	S	S	S	S	—	—	—	—	—	—
	0 005	S	S	S	S	S	S	—	—	—	—	—	—
	0 002	S	S	S	S	S	S	—	—	—	—	—	—
Tyrocidine	0 250	D4	D5	S	S	S	S	—	—	—	—	—	—
	0 100	D5	S	S	S	S	S	—	—	—	—	—	—
	0 050	D4	D4	D5	S	S	S	—	—	—	—	—	—
	0 025	D2	D2	D4	D4	D5	D8	—	—	—	—	—	—
Controls	0							D2	D4	D5	D4	D4	D5

* In this particular experiment all mice treated with gramicidin were alive and well when discarded 9 days after inoculation Usually a few scattered deaths are observed whatever the dose of gramicidin used for treatment.

† S = survival of the animal.
D = death Numeral indicates number of days elapsing between inoculation and death.

All these observations would indicate that, like other classical antiseptics, tyrocidine is essentially ineffective *in vivo* Surprisingly enough, however, crystalline preparations of this substance can exert a definite protective action against pneumococcus infections in mice This is illustrated in the following experiment

Mice were infected intraperitoneally with 10 000 fatal doses of Pneumococcus Type I within 15 minutes after infection they were treated intraperitoneally with varying amounts of gramicidin or tyrocidine diluted in distilled water

The results presented in Table II show that one single injection of 0 050 to 0 100 mg of tyrocidine administered intraperitoneally is sufficient to

protect mice against 10,000 fatal doses of pneumococcus, tyrocidine is however much less active than gramicidin, since the same protective effect could be obtained with 0 002 mg of the latter substance

Gramicidin and tyrocidine differ in many other biological properties, for instance 0 3 to 0 5 mg of gramicidin injected intraperitoneally is sufficient to kill a 25 gm mouse in 48 hours, 2 mg of tyrocidine is required for the same toxic effect, the latter substance therefore is less toxic than the former but it will be recalled that it is also much less effective against the Gram-positive bacterial cell both *in vitro* and *in vivo*

TABLE III

Hemolytic Activity of Gramicidin and Tyrocidine in Vitro

Bactericidal agent		1 cc of 10 per cent washed red cells—Hemolysis after incubation for the following lengths of time			
		15 min	3 hrs	8 hrs	24 hrs
	mg				
Gramicidin	0 400	−	−	−	−
	0 200	−	−	−	−
	0 100	−	−	−	−
	0 050	−	−	−	−
	0 020	−	−	−	−
Tyrocidine	0 400	++++	++++	++++	++++
	0 200	++++	++++	++++	++++
	0 100	+++	+++	+++	+++
	0 050	+	++	++	++
	0 020	−	+	+	+
Control	0	−	−	−	−

++++ = complete hemolysis
− = no hemolysis

Studies of the effect of gramicidin and tyrocidine on the physiological functions of the susceptible bacterial cells have also revealed profound differences in the mechanisms of action of the two substances, these studies will be reported later At this time, mention will be made only of the effect of the two bactericidal substances on the mammalian erythrocyte

Hemolytic Action of Gramicidin and Tyrocidine in Vitro—Rabbit erythrocytes were washed free of serum and resuspended in a volume of 5 per cent aqueous solution of glucose sufficient to give a concentration of cells corresponding to 1/10 that of the blood Graded dilutions of gramicidin and tyrocidine in 10 per cent glucose were added to the cell suspension and the mixtures incubated at 37°C Hemolysis readings were made after 15 minutes, 3 hours, 8 hours, and 24 hours incubation

As shown in Table III tyrocidine causes an immediate hemolytic effect which does not increase appreciably with prolonged incubation On the contrary, no hemolytic effect could be observed with gramicidin, even after 24 hours incubation

DISCUSSION

The antagonism exerted by certain types of microorganisms against other microbial species is a fact of common observation (12, 20) but the mechanism of the antagonistic action may vary so profoundly from one case to another that it hardly permits of any general systematic formulation "Antibiosis" (12) may be due, for instance, to competition for oxygen or other essential nutrients, to liberation into the culture medium of acidic or basic products which interfere with growth, to the production of other metabolites which may kill the cells, etc, etc The antagonistic action of certain aerobic sporulating organisms discussed in the present paper, offers on the contrary a fairly well defined entity From a great variety of sources (soil, sewage, manure, cheese, etc) strains can be isolated of aerobic sporulating bacilli, differing in morphological, cultural, and physiological characteristics, which all produce in peptone media an alcohol-soluble, water insoluble fraction endowed with bactericidal activity Among the first saprophytic, aerobic sporulating bacilli to be described, were those isolated by Duclaux (6) from Cantal cheese, on account of their origin, Duclaux gave to these organisms the generic name of *Tyrothrix* (now to be placed in the genus *Bacillus*) In 1925 Rosenthal (16, 18) showed that the strains of *Tyrothrix* isolated by Duclaux slowly release into the culture medium a substance endowed with lytic and bacteriostatic activity The antagonistic action recognized by Rosenthal was probably due to the alcohol soluble, water insoluble fraction described in the present and other reports The name tyrothricin has been proposed for this alcohol soluble, water insoluble fraction (10)

Tyrothricin has now been obtained by growing different species of aerobic sporulating bacilli on several media, (*a*) tryptone solution, a medium rich in tyrosine and tryptophane, (*b*) gelatin solution, a medium deficient in these aromatic amino acids, (*c*) synthetic media, consisting of mixtures of amino acids, with or without tryptophane and tyrosine The yields of tyrothricin have varied considerably on the different media with the different organisms It seems worth reporting that very large yields have been obtained by growing *Bacillus brevis* (strain B G) in a gelatin medium Since gelatin is deficient in aromatic amino acids, and since tyrothricin is rich in tyrosine and tryptophane, it is evident that the organism is capable of rapidly syn

thesizing large amounts of these aromatic amino acids It will be recalled also that many of the amino acids which constitute tyrothricin are of the unnatural *d*-type, since the *d*-amino acids are not present in gelatin, it appears that these substances are also synthesized by the bacillus in the course of its growth

Crude tyrothricin is bactericidal *in vitro* not only against Gram-positive microorganisms, but also against Gram-negative species Failure to recognize this fact in earlier publications was due to the following reasons (*a*) the activity of the crude product is very much greater against Gram-positive than against Gram-negative species, (*b*) the activity against Gram-negative bacilli is markedly inhibited in the presence of broth constituents, and all the earlier bactericidal tests were carried out directly in broth cultures

Tyrothricin, prepared from *Bacillus brevis* (strain B G) has yielded two crystalline products, the chemical nature of which has been outlined elsewhere (9, 10) One of these substances has been called gramicidin on account of its selective bacteriostatic and bactericidal effect against Gram-positive microorganisms The other substance is an organic base which has been called tyrocidine to recall the generic name of *Tyrothrix* and because the substance is rich in the amino acid tyrosine

In spite of their common origin and of the fact that both substances are polypeptides, gramicidin and tyrocidine differ not only in certain chemical properties, but also in biological activity Gramicidin is effective only against Gram-positive microorganisms, tyrocidine, when tested in buffer solution in the absence of broth, affects both Gram-positive and Gram-negative species Tyrocidine causes immediate hemolysis of washed red cells, whereas gramicidin has no hemolytic effect Tyrocidine also causes lysis of many bacterial species, there is definite evidence, however, that the lytic effect in this case is not a direct one, but is only a secondary autolytic process which follows upon death of the cell (2)

Although the effect of gramicidin is to some extent inhibited by the presence of peptones and serum, this inhibitory effect is especially marked in the case of tyrocidine, in fact, it is very difficult to recognize any effect of tyrocidine on Gram-negative bacilli when these organisms are suspended in peptone solutions

It will be shown elsewhere that tyrocidine immediately destroys the metabolic activity not only of bacterial but also of animal cells This effect can be recognized by the immediate loss of oxygen uptake, of acid production, of reducing ability On the contrary these essential metabolic functions are respected by gramicidin even in the case of the most susceptible bacterial cells

All available evidence, therefore, indicates that tyrocidine behaves like a general protoplasmic poison, whereas the effect of gramicidin is of a much more subtle nature In fact it will be shown elsewhere that the effect of gramicidin is to some extent reversible For instance staphylococci "killed" with gramicidin and which are unable to grow on meat infusion peptone media can be made to grow in the presence of certain tissue components (1)

Since gramicidin is not a gross protoplasmic poison, and since it is less inhibited by peptones than are most antiseptics, it becomes easier to understand why under certain conditions it retains much of its activity in the presence of animal tissues In fact, gramicidin, when applied locally at the site of the infected area, does exhibit a definite activity against infection with pneumococci and streptococci (9, 11, 12) It appears, however, that gramicidin is almost completely inactive against systemic infection when injected intravenously (4) Whether this ineffectiveness is due to physical properties which prevent diffusibility of the substance throughout the tissues or whether it is due to the inhibitory effect of tissue components upon its activity, cannot be decided at the present time

Tyrocidine, although inactive against infection with Gram negative bacilli, appears to exhibit definite activity against pneumococcus infection in mice The results reported in Table II have been obtained with preparations recrystallized several times and can hardly be explained by a contamination of tyrocidine with gramicidin Tyrocidine is much less active than gramicidin against pneumococcus infections in mice, on the other hand, when tested *in vitro* against these same microorganisms resuspended in buffer solutions, tyrocidine is almost as active as gramicidin This discrepancy appears of special interest since it offers a concrete example of two substances having a common origin, definite similarity in chemical structure, but differing widely in "chemotherapeutic" action It is hoped that a comparison of the chemical structure of the two substances, and a knowledge of the mechanism of their physiological action against bacterial and tissue cells, may throw light on some of the factors which govern the effectiveness of antiseptic agents in the animal body

SUMMARY

Several species of aerobic sporulating bacilli recently isolated from soil, sewage, manure, and cheese, as well as authentic strains obtained from type culture collections, have been found to exhibit antagonistic activity against unrelated microorganisms

Cultures of these aerobic sporulating bacilli yield an alcohol soluble, water insoluble fraction,—tyrothricin,—which is bactericidal for most Gram positive and Gram negative microbial species

Two different crystalline products have been separated from tyrothricin One, which may be called tyrocidine, is bactericidal *in vitro* for both Gram-positive and Gram-negative species, the other substance, gramicidin, is effective only against Gram-positive microorganisms In general, tyrocidine behaves like a protoplasmic poison and like other antiseptics, loses much of its activity in the presence of animal tissues Gramicidin on the contrary exerts a much more subtle physiological effect on the susceptible bacterial cells and, when applied locally at the site of the infection, retains *in vivo* a striking activity against Gram-positive microorganisms

Addendum —Heilman and Herrell (*Proc Soc Exp Biol and Med*, 1941, 46, 182) have recently described a marked hemolytic effect of gramicidin in tissue culture, whereas, in the experiments reported in the present paper, no hemolysis was observed when washed sheep red cells were resuspended in isotonic glucose solution We have now established that gramicidin does indeed cause a slow hemolysis of erythrocytes resuspended in buffer or saline solutions, but the addition of small amounts of glucose to the system is sufficient to prevent any hemolytic action

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A VIRUS FROM CASES OF ATYPICAL PNEUMONIA

RELATION TO THE VIRUSES OF MENINGOPNEUMONITIS AND PSITTACOSIS*

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PLATE 32

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The isolation of three different etiologic agents from atypical bronchopneumonias has recently been reported Dyer, Topping and Bengston (1) have described an outbreak of pneumonitis apparently resulting from laboratory infections in which the rickettsiae of American Q fever were shown to be the cause Weir and Horsfall (2) have reported infection of the mongoose with a filterable agent from cases of atypical pneumonia In 1939, Stokes Kenney and Shaw (3) isolated from the nasopharyngeal washings of a patient with bronchopneumonia by inoculation of ferrets and mice a virus which produced pneumonia and encephalitis in experimental animals

In describing the clinical manifestations of atypical bronchopneumonias some authors (4, 6) have considered psittacosis in the differential diagnosis A comparison of the cases described by Reimann (4) Kneeland and Smetana (6), and Longcope (7) with cases of recognized psittacosis (8) in some instances reveals clinical similarities However, the cases described by other authors (1 2 16) were milder and showed less similarity to psittacosis Apparently transmission of the infectious agent from one human being to another occurs quite readily under certain conditions In some outbreaks several cases have occurred in a series indicating repeated direct human to-human infections

This paper will describe the isolation of a psittacosis like virus from four cases of atypical pneumonia The properties of this agent differed in certain respects from those of the ordinary strains of psittacosis The new strain of virus is antigenically related to, but not identical with, the strain of virus isolated by Francis and Magill (5) and named by them the virus of meningopneumonitis Both of these strains are also antigenically related to psittacosis virus from parrots

Materials and Methods

The six cases studied were all connected epidemiologically A brief summary is given to make clear the sources of material Case 1 entered a hospital in San Francisco on Mar 8 1940 after having been ill for approximately 1 week with an influenza like disease

*The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation and in cooperation with the California State Department of Public Health

Examination revealed a bronchopneumonia with pleurisy and effusion On Mar 18, the patient died The cause of death was given as influenzal bronchopneumonia No autopsy was performed Contact with parrots or other psittacine birds could not be demonstrated by questioning the family of the patient [1] Cases 2, 3, and 4 were nurses who took care of case 1 and contracted a similar illness with pneumonia All became ill between Mar 25 and 27, and two died on Apr 7 Specimens of lung, liver, and spleen obtained at autopsy from cases 2 and 3 were sent to our laboratory [2] Case 4 recovered after a severe illness Cases 5 and 6 were laboratory workers who contracted a disease similar to that of the previous cases, apparently as a result of infection with the agent isolated from the lungs of cases 2 and 3 Case 5 became ill on Aug 11, 1940, and recovered after an illness lasting approximately 3 weeks Case 6 became ill on Aug 16 and recovered after a prolonged illness of about 6 weeks Throat washings, sputum, and blood from these two cases were studied

In all six patients the disease was characterized by an onset with influenza-like symptoms lasting 2 to 6 days accompanied by gastrointestinal complaints, severe headache, and in one case signs of meningeal irritation All cases developed a bronchopneumonia of varying extent which was not detected until several days after the onset of the illness The temperature was high and usually continuous and the pulse relatively slow One case had an intermittent fever, then an afebrile period followed by a mild relapse and later by a migrating arthritis The white blood count was normal or only slightly elevated Sputum examinations revealed no significant bacteria Clinically the disease was similar to many of the cases of atypical pneumonia described in the literature (4, 6, 7)

Animal Inoculation —Mice and Syrian hamsters (*Cricetus auratus*) were inoculated intranasally, intracerebrally, and intraperitoneally, guinea pigs intracerebrally and intraperitoneally, rabbits intraperitoneally, and Java ricebirds (*Munia oryzivora*) intramuscularly Intracerebral and intranasal inoculations were done under ether anesthesia

Complement Fixation Tests —Antigen was prepared from the infected lungs of mice by grinding with alundum and saline The resultant suspension was centrifuged at 1,500 R P M to remove large particles It was used in the tests unheated at a concentration of 2 per cent wet mouse lung Normal control antigen was similarly prepared from normal mouse lungs The test in other details was identical with that used for influenza

Isolation of Virus by Intranasal Inoculation of Mice

Intranasal inoculation of twelve mice with unfiltered suspension of lung obtained at autopsy from cases 2 and 3 resulted in no apparent illness, but when the animals were sacrificed on the 7th day many small round bluish grey focal lesions were found in the lungs of all mice The appearance of these lesions in tissue section under low and high magnification is shown in Figs 1 and 2 No similar lesions were seen in the lungs of mice which had been inoculated with various other materials or in normal

[1] Epidemiological investigations were done under the direction of Dr J C Geiger, director of public health for the city of San Francisco

[2] Through the kindness of Dr A M Moody and Dr W T Cummings of the Southern Pacific Hospital

mice The lesions were entirely different from those produced by the vi ruses of influenza and mouse pneumonia

Culture of the original human material showed a *Staphylococcus aureus* in the lungs of one case and nothing in the other Cultures from the mouse lungs gave no growth on the ordinary bacteriological culture media Further passage from the lungs of the first mice increased the virulence of the

TABLE I

Results of Inoculating Mice with Material from Four Cases of Atypical Pneumonia

Case No	Material	Day of illness	Mice inoculated intranasally	Mice inoculated intraperitoneally	Interval of intraperitoneal passage
					days
		13th	Pos 1*	Neg 3	7, 5 19
2	Lung	13th	Neg 2	—	
	Liver	13th	Neg 2	—	
	Spleen				7 5, 19
		14th	Pos. 1†	Neg 3	
3	Lung	14th	Neg 3	—	
	Liver	14th	Pos 2	—	
	Spleen				
		2nd	Neg 4	—	7 20 21
5	Throat washing	5th	Neg 3	Doubtful 3‡	7 7 8 14
	Blood	10th	Pos. 2	Neg 4	8 20 33
	Sputum	13th	Pos 2	Doubtful 2‡	
	Sputum			—	
		2nd	Neg 4	Neg 4	7, 7, 7 21
6	Throat washing	8th	Pos 1	Neg 3	21 21 21
	Sputum	12th	Pos 1	—	
	Sputum	36th	Neg 1		

Explanation of columns 4 and 5 Pos 1 means definite pathology seen on first and subsequent passages Neg 4 means no pathology or symptoms in mice after four passages

* Repeated twice with original material positive both times on first passage

† Repeated twice with original material positive once on first passage

‡ Intranasal passage of livers and spleens of mice showing peritoneal exudate gave no lung lesions

infectious agent until on the fourth passage the mice developed sticky eyes, ruffled fur, bubbling respiration, and died within 2 to 3 days

A similar result was obtained with sputum taken on the 10th and 13th days of illness from case 5 and on the 8th and 12th days of illness from case 6 No significant bacteria were found The results of mouse inoculation are summarized in Table I

The same material which produced lung lesions in the first or second passage after intranasal inoculation of mice failed to produce any significant illness or pathology after intraperitoneal inoculation Repeated passage

of suspensions of livers and spleens of mice by the intraperitoneal route at various intervals of time had no effect except that when passage was done at an interval of about 20 days, enlarged spleens and slight peritoneal exudates were noted in some of the mice However, further passages did not increase the virulence of the agent and subinoculation of liver and spleen suspensions into mice by the intranasal route did not produce characteristic lung lesions

As shown in Table I, negative results were obtained by intranasal inoculation of mice with suspensions of liver from two cases, spleen from one case, blood from one case, and throat washings taken on the 2nd day of illness from two cases From the spleen of one case virus was isolated

Properties of the Virus

The viruses isolated from cases 2 and 3 were apparently identical and have been studied so far in the greatest detail In subsequent sections of this paper this strain will be designated by the letters S-F A strain of meningopneumonitis virus sent us by Dr Thomas Francis, Jr , for comparison with our strain will be designated M P -F97

In impression smears from the lungs of mice dying 2 to 3 days after intranasal inoculation with strains S-F or M P -F97, elementary bodies which were stained red by Machiavello's method were seen The bodies were very similar in size and appearance to the Levinthal-Coles-Lillie bodies of psittacosis They were also seen occasionally in peritoneal and meningeal exudates after intracerebral or intraperitoneal inoculation Similar bodies were not seen in the lungs of normal mice nor in mice inoculated with the viruses of influenza or mouse pneumonia

After centrifugation of suspensions of infected mouse lung containing the strain S-F at 5,000 R P M for 1 hour, practically all of the infectious material was sedimented Attempts to filter the agent through Berkefeld V and Seitz filters have so far been unsuccessful The large particle size of the virus was indicated by these experiments

Pathogenicity for Animals

Mice Intranasal Inoculation—With the strain S F, the appearance of lung lesions in mice depended upon the stage of the disease and the amount of virus inoculated Dilutions of 10^{-3} to 10^{-4} did not produce a uniformly fatal disease and small round grey foci were seen With larger doses the foci became confluent and solid or patchy greyish pink lesions were produced In mice dying acutely after intranasal inoculation of 10 per cent lung suspensions, the lungs were completely consolidated, deep red, and very edematous There was a small amount of sticky pleural exudate The various lesions observed were not dissimilar from those seen in mice inoculated intranasally with parrot

strains of psittacosis virus (9 10) The virus has been carried through fifteen intranasal passages in mice without apparent change

Intracerebral Inoculation —After the strain S F had been carried for four passages by intranasal inoculation, mice were inoculated intracerebrally with a bacteriologically sterile lung suspension After a few intracerebral passages a dilution of 10^{-2} of mouse brain was uniformly fatal to mice in 4 days Higher dilutions up to 10^{-5} killed part of the mice in 6 to 10 days with paralysis of varying degree preceding death

TABLE II

Lesions and Survival of Virus in Mice after Intraperitoneal Inoculation

Mice inoculated intraperitoneally with strain	Dilution	Killed	Peritoneal exudate	Enlargement of spleen and liver	Result of subinoculation of livers and spleens to mice	
					Intranasally*	Intracerebrally
		day				
S-F	10^{-1}	3	+ watery	0	++++ +++ ++	—
S-F	10^{-1}	6	+ sticky	0	++ + ±	—
S-F	10^{-1}	9	0	0	± 0 0	S S S
S-F	10^{-1}	12	0	0	0 0 0	S S S
S-F	10^{-1}	18	0	0	0 0 0	9 S, S
S-F	10^{-1}	25	0	0	0 0 0	S S, S
S-F	10^{-1}	30	0	0	0 0 0	S S, S
M P F97	10^{-2}	3	+++ sticky	0	7 7, +++	—
M P F97	10^{-2}	6	++ fibrin	±	5 5 7	—
M P F97	10^{-2}	9	++ fibrin	+	9 +++ ++	6, 6, 7P
M P F97	10^{-2}	12	+++ fibrin and ascites	+	8 10 10	5P 5 7
M P F97	10^{-2}	18	+ fibrin	±	9 9 10	4 4 4
M P F97	10^{-2}	25	±	±	7 9 ++++	5 5 7P
M P F97	10^{-2}	30	+	±	++++ ++++, +++	5 5 5

* Explanation of symbols Figures in 6th and 7th column represent day of death of mice. Plus signs under intranasal inoculation indicate lung lesions in mice which survived 10 days. P after day of death signifies paralysis of hind legs observed before death S means mice survived without symptoms 0 means survival without lesions

The strains M P F97 and S F both produced an identical type of pneumonia without encephalitis or meningitis after intranasal inoculation and both gave meningitis and paralysis of the hind legs without pneumonia after intracerebral inoculation

Intraperitoneal Inoculation —Over one hundred mice were inoculated intraperitoneally with 10 per cent lung or brain suspensions of the strain S-F from the later passages which were highly virulent by the intranasal or intracerebral routes Of the few animals which died none showed definite lesions in the abdominal organs or peritoneal exudate and the deaths were considered to be non specific

Definite differences between the strains S F and M P F97 were observed after intraperitoneal inoculation of mice Thirty mice were inoculated intraperitoneally with each strain and killed at various intervals The results presented in Table II show that the strain S F produced only a slight peritoneal exudate during the first week, but nothing

later except occasionally a slightly enlarged spleen During the first 6 days some of the mice appeared ill and subinoculation of the livers and spleens by the intranasal route produced lung lesions characteristic of the virus, but after 9 days the virus was no longer detectable by intranasal or intracerebral inoculation On the other hand, the strain M P -F97 produced illness or death with a heavy peritoneal exudate which was later followed by a deposit of fibrin, enlargement of the liver and spleen, and development of ascites A carrier state was set up in mice which recovered from the acute infection and virus was detectable by intranasal or intracerebral subinoculation of liver and spleen for as long as 30 days Paralysis was seen in about 5 per cent of mice inoculated intraperitoneally with either strain

Intraperitoneal passages with the strain S-F were started by inoculation of lung suspension from the 8th, 10th, and 13th intranasal passages The virus was then carried by intraperitoneal passage of liver and spleen suspensions at intervals of 7 days for eight passages Although the virulence of the agent was not appreciably increased, intranasal inoculation of suspensions of liver and spleen from the last passage produced characteristic lesions in the lungs of mice, indicating that the virus was successfully carried by this method

Subcutaneous Inoculation —Mice inoculated subcutaneously with the strain S-F showed no definite lesions except a slight enlargement of the regional nodes This is in contrast to the marked induration and suppuration which follows subcutaneous inoculation of the strain M P -F97

Guinea Pigs Intracerebral Inoculation —The strain S-F, inoculated intracerebrally, caused fever, ruffled fur, emaciation, and sometimes death A meningitis with hyperemia of the brain, and occasionally small pneumonic patches in the lungs, were noted at autopsy There were no pathological changes in the liver and spleen

Intraperitoneal Inoculation —The strain S-F produced, in guinea pigs inoculated intraperitoneally, a prolonged febrile illness lasting 8 to 14 days with extreme emaciation and temperatures ranging from 104°F to 105 3°F Some animals died in 7 to 14 days

Passage of the virus intraperitoneally in guinea pigs resulted in a decrease in virulence during the first few passages

Lesions observed in animals inoculated by this route included a fibrinous peritoneal exudate, hyperemia of the peritoneal surfaces, sometimes hepatic necrosis, patchy lesions in the lungs, and slight enlargement of the spleen These lesions tended to regress after about 2 weeks and in animals sacrificed after 6 weeks the abdominal organs were negative, but grey patches probably representing healed lesions were seen in the lungs Virus was recovered from the lungs, liver, and spleen of animals in the acute stages of the disease by intranasal inoculation of mice

Inoculation of the strain M P -F97 into guinea pigs by the intraperitoneal route resulted in a mild transient illness No lesions were seen in the abdominal organs except a slight watery peritoneal exudate and reddening of the peritoneal surfaces From these results it appears that the strain S-F is much more virulent for guinea pigs than the strain M P -F97

Syrian Hamsters Intranasal Injection —Strains S-F and M P -F97, intranasally injected, produced in Syrian hamsters a pneumonia similar to that observed in mice Elementary bodies were abundant in smears of the lungs stained by Machiavello's method

Intracerebral Injection—The two strains injected intracerebrally resulted in an illness manifested by rough fur, weakness ataxia coma and death in 3 to 7 days No definite paralysis was noted The principal lesion was a meningitis

Microscopic Pathology

Study of sections of the lungs and brains of animals receiving the strains S F and M P -F97 intranasally and intracerebrally revealed pathological changes identical with those described by Francis and Magill (5) (See Figs 1 and 2)

The livers of a number of mice receiving the two strains intraperitoneally were examined microscopically The cells in certain areas of the livers appeared vacuolated and infiltration with leucocytes was observed Occasionally, only small nests of mononuclear cells were seen In the livers of mice receiving the strain S F, areas of degeneration were less marked and less frequent than in the mice inoculated with the strain M P F97, and the watery peritoneal exudate contained a moderate number of large mononuclear cells and lymphocytes, but elementary bodies were seldom demonstrated The spleens showed no striking pathology

Pathogenicity for Java Ricebirds

The purpose of these experiments was to determine if the strains under investigation showed the same degree of infectiousness for Java ricebirds as do the strains of psittacosis virus from parrots and other psittacine birds (15)

Java ricebirds were inoculated in the pectoral muscle with 10 per cent mouse brain and mouse lung suspensions containing the strains S-F and M P F97 Mice inoculated intranasally at the same time died with typical lung lesions The results are presented in Table III Two of the ricebirds (Nos 1 and 2) which received the strain S F died in 8 and 14 days respectively without definite pathology except for the presence of moderate diarrhea in one bird and some fluid in the lungs and pleural cavity of the other Virus was demonstrated in the organs of these birds by intranasal inoculation of suspensions of lung liver and spleen into mice Mice inoculated intraperitoneally remained well Four of the ricebirds inoculated with the strain S-F survived for 28 days at which time they were killed and autopsied Moderately enlarged spleens were seen in all four birds and one (No 6) had questionable lesions in the liver and lungs The presence of active virus could not be demonstrated by subinoculation of livers lungs, and spleens into mice

The ricebirds receiving the strain M P F97 died in 9 to 15 days Nos 8 to 12 inclusive had hepatic necrosis enlarged spleens patches of consolidation in the lungs and diarrhea In the organs of all birds virus was readily demonstrated by subinoculation of mice by the intranasal and intraperitoneal routes

Serological Tests

Sera obtained from cases 4, 5, and 6 were tested by complement fixation against the strains S F and M P F97 From the results in Table IV, it

The serum from case 4 was first tested by complement fixation with heated psittacosis tissue culture antigen by Dr K F Meyer and Miss B Eddie (11) at the laboratories of The George Williams Hooper Foundation This serum gave a high titer with psittacosis antigen and Dr Meyer very kindly sent us a portion of it which was tested with the results shown in Table IV Subsequently the serum specimens from cases 5 and 6 were also tested in Dr Meyer's laboratory These tests showed an increase in the titer of complement fixing antibodies with the psittacosis antigen, similar to that observed with the strains S F and M P F97

Production of complement fixing antibodies in guinea pigs and mice with the strain S F was irregular However, the serum of animals immune to the strain M P F97, although failing to give definite fixation with unheated mouse lung antigens from this strain did give titers of 1 16 or above with the heated antigen from psittacosis virus in tissue culture Studies on this phase of the problem have not yet been completed

No satisfactory method has yet been developed of demonstrating in the serum of immune animals neutralizing antibodies to the strain S F The method used by Francis and Magill (5) could not be applied to our strain because of its low virulence for mice by the intraperitoneal route

Active Immunity

Homologous Immunity—Mice which had received a single intraperitoneal inoculation with the strain S F were not immune to infection by the intranasal route, but did resist infection by the intracerebral route This is in agreement with the results of Francis and Magill (5) A single intranasal dose of virus sufficient to produce small lung lesions without killing any mice (about 1/10 M L D) did not produce definite immunity to reinoculation with approximately 10 M L D by the same route Similarly, single subfatal doses of virus given intracerebrally often failed to produce solid immunity to intracerebral inoculation of 10 M L D However, larger amounts of virus inoculated intranasally, although fatal to some of the mice, usually produced solid immunity in the survivors to reinoculation by the same route When multiple inoculations were done, starting with 1/10 M L D and increasing the amount ten times at each successive injection, a much more solid immunity was produced

Guinea pigs which had recovered from intraperitoneal infection with the strain S F showed no rise in temperature or other signs of illness after a second intraperitoneal inoculation with this strain

Cross Immunity—Although it was difficult to produce cross immunity to M P F97 with the strain S F by intranasal inoculation, the converse was not true Mice receiving one subfatal dose of the strain M P F97 intranasally were definitely immune to the strain S F when tested 1 month later Mice which had received two intranasal inoculations with the strain S F did not resist infection by the intranasal route with 100 M L D of the strain M P -F97, although animals from this same group were immune to the homologous strain

The results presented in Table I strongly suggest that the strains isolated by us came from human material since in three out of four cases positive results were obtained in all of the mice of the first passage This view is supported by the demonstration of an increase in complement fixing antibodies in cases 5 and 6 (Table IV) This increase was associated with an illness similar to that of cases 2 and 3 and apparently resulted from laboratory infection A similar strain of virus was isolated from the sputum The more remote origins of the strain S F are obscure History of contact with psittacine birds within a period of 1 month before onset was not obtained in any case

On the basis of the results published by Francis and Magill (5), it appears that the strain M P F97 and other antigenically related strains of the virus of meningopneumonitis isolated by these authors came either from ferrets or from the throats of human beings with a disease clinically resembling epidemic influenza[1] The failure to demonstrate neutralizing antibodies to the virus in the serum of the human cases does not constitute decisive evidence of the non human origin of the virus, because it is known that infection with the virus of psittacosis, to which the virus of meningopneumonitis is antigenically related, does not regularly stimulate the production of neutralizing antibodies (12)

The possibility that the strain S F came from a source other than psittacine birds is suggested by certain peculiarities of the strain namely, (*a*) its low virulence in the peritoneum of mice as compared with its relatively high virulence in the lungs or brain, (*b*) the relatively low virulence for Java ricebirds which readily become infected with psittacosis virus by contact with diseased parrots, (*c*) the failure of the strain to produce a carrier state in mice and birds which recover With respect to these three properties, the strain M.P F97 appears to be more closely related to true psittacosis virus than the strain S F Both strains seem to have a relatively high pneumotropism Hornus (*9*) has shown that psittacosis virus is not modified in its intraperitoneal virulence for mice by repeated intranasal passage Paralysis in mice after intracerebral or intraperitoneal inoculation is caused not only by these two strains, but also occasionally by strains of psittacosis virus (8) from parrots

It is possible that an as yet unknown vector may be responsible for the spread of these atypical strains of virus Bedson (13) has recently described

[1]Since this paper was prepared for publication Dr Francis has informed us that a majority of the cases studied in the epidemic referred to showed an increase in neutralizing antibodies to the virus of influenza B (17)

the infection of human beings with psittacosis virus from fulmar petrels, a previously unsuspected source Pinkerton and Swank (14) have reported the isolation of a psittacosis-like virus from thiamin-deficient pigeons This agent was pathogenic for mice by intracerebral inoculation, but intraperitoneal or subcutaneous inoculation was without effect Finally, it is possible that one or both of the agents which we have studied are actually representatives of a group of psittacosis-like viruses that have become adapted to man with an increased infectiousness for the respiratory tract and a diminished virulence for birds and for mice inoculated by the intraperitoneal route

SUMMARY

From the lungs of two fatal cases and from the sputum of two non-fatal cases of atypical bronchopneumonia, a psittacosis-like virus was isolated by direct intranasal inoculation of mice Intraperitoneal injection of the same human material into mice gave negative results

The virus has a relatively high virulence for mice by intranasal or intracerebral inoculation, but does not kill after intraperitoneal inoculation

Its virulence for Java ricebirds is low and it fails to produce a carrier state in mice and birds

Two cases showed an increase in complement-fixing antibodies to the new virus and to psittacosis

The virus is antigenically related to the viruses of meningopneumonitis and psittacosis by complement fixation and by active immunity tests in mice

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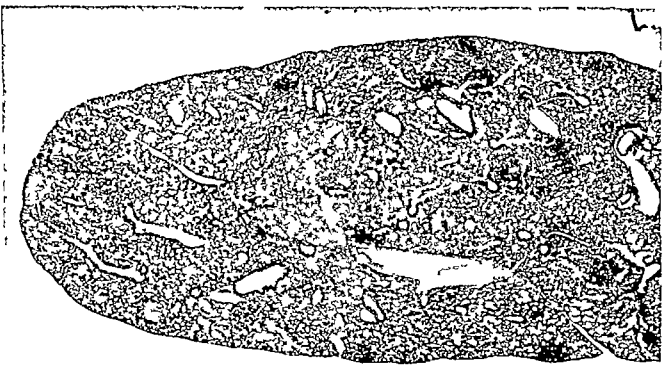
EXPLANATION OF PLATE 32

FIG 1 Section of mouse lung taken from second intranasal passage of lung material from case 2 Hematoxylin and eosin There are many small localized areas of infiltration which appear darker than the surrounding lung tissue × 22

FIG 2 Same, showing detail of one of the foci In the center is a dense accumulation of mononuclear cells with a few small pockets of polymorphonuclear leucocytes The walls of the adjacent alveoli are swollen and there is some fluid in the air spaces There is also some perivascular infiltration, but no striking change in the columnar epithelium of the bronchiole × 251



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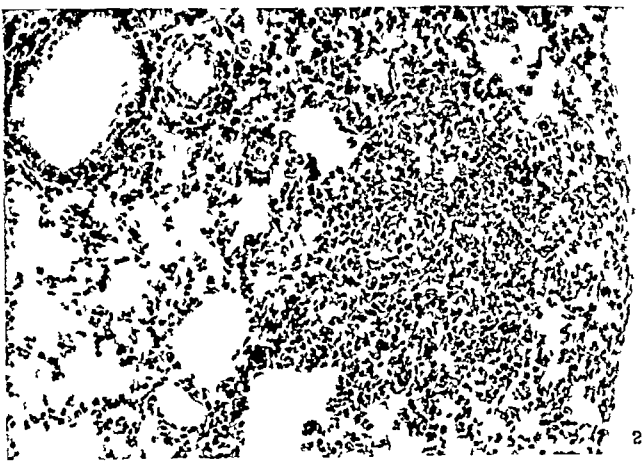

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THE ISOLATION OF AN O SPECIFIC SUBSTANCE FROM GASTRIC JUICE OF SECRETORS AND CARBOHYDRATE LIKE SUBSTANCES FROM GASTRIC JUICE OF NON SECRETORS

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The nature of the blood group specific substances has been studied by many investigators, among them Brahn and Schiff (urine and saliva) (1, 2), Brahn Schiff and Weinmann (pepsin) (3) Freudenberg Eichel and Dirscherl (urine) (4) Freudenberg and Westphal (peptone) (5), Jorpes and Norlin (urine) (6) Hallauer (blood) (7) and Meyer Smyth, and Palmer (pig gastric mucosa) (8) Some of these authors have stressed the carbohydrate nature of the substances with which they were working Landsteiner (9) isolated from horse saliva a purified carbohydrate like substance exhibiting strong A potency The original material used for the isolation of the group specific substances seems to play an important rôle in these investigations Their concentration in the tissue cells and body fluids varies considerably Putkonen (10) gives the following relative figures in regard to the concentration of group specific substances in various materials of human origin Saliva, 108 to 1024 units blood 8 to 32 units, urine 2 to 4 units In a thorough study of the quantitative occurrence of water soluble group specific substances in tissues and secretions Friedenreich and Hartman (11) agree principally with Putkonen's findings

In a previous communication (12), the isolation of a carbohydrate like substance from human gastric juice of patients belonging to group B has been reported This substance exhibits strong B potency and is as active as the A preparations obtained according to methods recently employed (*cf* Landsteiner and Chase (13), Landsteiner and Harte (14), Goebel (15)) The investigations to be reported here deal with the question as to whether it is possible to isolate an O substance from gastric juice of human beings of group O According to the original belief human cells belonging to group O are characterized by the absence of the A and B substances rather than by the presence of a specific O factor Some normal animal sera, particularly beef sera, as well as certain immune sera, agglutinate human red blood cells of group O in somewhat higher dilutions than cells of other groups (*cf* Schiff (16)) The treatment of such normal beef sera with A_1B cells removes agglutinins acting on the blood cells of groups other than

groups The absorption was repeated if red blood cells other than O still were agglutinated too strongly A potent anti-O reagent usually agglutinates, at least slightly, human blood cells belonging to the various groups If the absorbed serum was found to be satisfactory, it was dried in amounts of 1 to 10 cc per ampoule or kept in the refrigerator in the presence of 0 01 per cent merthiolate

TABLE I

Agglutination of Human Red Blood Cells of Various Groups by Normal Beef Serum

Dilutions of beef sera	Beef sera				
	2	3	7	11	12
Group O Cell Suspension					
(1) 1 10	++	++	+++	+++	+++
(2) 1 20	+	+	+++	+++	+++
(3) 1 40	−	±	++	+++	+
(4) 1 80	−	−	+	++	+
(5) Saline	−	−	−	−	−
Group A Cell Suspension					
(1) 1 10	++	+++	+++	+++	++
(2) 1 20	+	++	+++	+++	++
(3) 1 40	±	++	++	++	+
(4) 1 80	−	+	+	++	±
(5) Saline	−	−	−	−	−
Group B Cell Suspension					
(1) 1 10	+	++	+++	++	++
(2) 1 20	±	++	+++	+	+
(3) 1 40	−	+	+	±	+
(4) 1 80	−	±	+	±	±
(5) Saline	−	−	−	−	−

− = no agglutination
± = faint agglutination
+ = slight agglutination
++ = marked agglutination
+++ = strong agglutination
++++ = very strong agglutination

Tests of Beef Sera —The agglutination of human red blood cells belonging to different blood groups by various normal beef sera is shown in Table I The experiment was carried out in the following way —

Decreasing amounts of beef serum, volume 0 2 cc , were mixed with 0 2 cc of a 1 per cent suspension of human red blood cells belonging to the groups O, A, and B respectively These mixtures were kept at room temperature for 10 minutes and then centrifuged for 1 minute at medium speed The tubes were then shaken up slightly and the degree of agglutination determined

Table I shows that different types of normal beef sera occur although the differences may not be too marked (*cf* Witebsky and Okabe (21), Schiff and Sasaki (22), Sasaki (20),

Friedenreich and Zacho (18), Dahr (23)) Some beef sera contain only weak agglutinins for all types of human cells while the titers of others are definitely higher Some show a preference for red blood cells of group A An additional difficulty consists in the fact that different blood specimens even of the same group, show considerable variation in the degree of agglutinability Among the beef sera given in Table I serum 11 was selected and absorbed with cells belonging to the group A_1B It should be stated in this connection that not all cells of group A_1B are equally suitable for absorption for reasons that need further clarification

Table II shows an experiment demonstrating the anti O agglutination by beef serum 11 after its treatment with A_1B cells The experiment itself was carried out in the following way:—

Decreasing amounts of absorbed beef serum 11, volume 0 2 cc were mixed with 0 2 cc of (*a*) 1 per cent suspension of human A_1B cells (*b*) 1 per cent suspension of human O cells The first tube contained a 1 2 dilution of the absorbed beef serum corresponding

TABLE II

Agglutination of Human Red Blood Cells by Means of Absorbed Normal Beef Serum

Absorbed beef serum 11	Group AB cell suspension	Group O cell suspension
(1) 1 2	+	+++
(2) 1 4	±	+++
(3) 1 8	−	+++
(4) 1 16	−	++
(5) 1 32	−	++
(6) 1 64	−	+
(7) 0	−	−

to a 1 4 dilution of the original native serum The test tubes were kept at room temperature for 15 minutes and then centrifuged at a medium speed for 1 minute The resulting agglutination was observed after the tubes had been shaken up In the case of O agglutination the tubes should be shaken only slightly Too violent shaking might break up the agglutinated cells completely

The agglutination of human red blood cells of group O by means of the absorbed beef serum 11 is quite definite when compared with the agglutination of A_1B cells Beef serum 11 was the strongest anti O serum found in over one hundred blood specimens examined so far for this purpose However it is possible to use beef serum that does not contain such a strong anti O agglutinin as beef serum 11 Experiments to be referred to in this paper have been carried out not only with beef serum 11 but also with other anti-O beef sera

EXPERIMENTAL

Large amounts of group specific substances are present in the saliva and gastric juice of human beings As far as we know, these secretions contain the highest concentration of group specific substances occurring within the human body There are, however, certain persons whose saliva is more or

less free of group specific substances Roughly 20 to 30 per cent of all people belong to the group of "non-secretors" The others constitute the large group of "secretors" (Putkonen (10), Lehrs (24), Schiff and Sasaki (22)) The demonstration of the group specific substances in saliva and gastric juice is accomplished by means of the "inhibition of agglutination" test This inhibition is perfectly group specific, inasmuch as secretions containing the A substance inhibit the potency of the isoagglutinin anti-A while B-containing specimens counteract the isoagglutinin anti-B Among the individuals belonging to group O, secretors as well as non-secretors are

TABLE III

Agglutination of Human Red Blood Cells Belonging to Group O by Means of Absorbed Beef Serum Anti O after Treatment of the Latter with Specimens of Gastric Juice and Saliva Respectively

Dilutions of gastric juice and saliva	I Gastric juice specimens from			II Saliva specimens from		
	Patient 1 Group O	Patient 2 Group O	Patient 3 Group A	Patient 1 Group O	Patient 2 Group O	Patient 3 Group A
(1) Undiluted	+	−	−	+++	X	X
(2) 1 3	+	−	−	+++	−	−
(3) 1 9	++	−	−	+++	−	−
(4) 1 27	++	−	+	+++	+	+
(5) 1 81	+++	+	+	+++	+	+
(6) 1 240	+++	+	+	+++	+	+
(7) 1 720	+++	++	++	+++	+	+
(8) 1 2200	+++	+++	++	+++	+	+
(9) 0	+++	+++	+++	+++	+++	+++

X = missing

found However, inhibitory potency toward the O agglutination is not only exhibited by specimens from O individuals but is present more or less in those of secretors belonging to other blood groups (*cf* Sasaki (20)) Table III shows an experiment in which the inhibitory potency of gastric juice specimens of three different individuals toward the O agglutination is compared with that of saliva The experiment itself was carried out in the following way —

Specimens of gastric juice and saliva were heated in a boiling water bath for 10 minutes immediately after being collected and then centrifuged The clear supernatant fluid was used in the following experiment

Decreasing amounts of gastric juice (I) and saliva (II) respectively, volume 0 2 cc , were mixed with 0 1 cc of an absorbed beef serum anti O and kept at room temperature for 15 minutes Then 0 1 cc of a 1 per cent suspension of human red blood cells of group O was added The mixtures were again kept at room temperature for about 15 minutes and then centrifuged The resulting agglutination can be seen from Table III

Table III shows that the saliva and gastric juice specimens obtained from patient 1 do not, or only slightly, inhibit the agglutination of O cells by anti O beef serum In contradistinction, the saliva and gastric juice specimens of patients 2 and 3 exhibit definite inhibitory activity Attention is drawn to the fact that the first two patients belong to group O and the third patient to group A The experiment clearly indicates the existing difference between secretors and non secretors recognizable even as far as group O is concerned However, the inhibition of O agglutination by secretions containing the O factor is much weaker than the inhibition of agglutination of A and B cells by means of secretions containing the A and B factors respectively The difference in degree of inhibition is considerable It must be kept in mind, of course, that the inhibitory power of specimens from different individuals varies a great deal This variation rarely creates any diagnostic difficulties as far as the specimens from A or B patients are concerned because the inhibitory potency in these instances is usually marked But in the case of the O agglutination it is our experience that difficulties may be encountered in determining whether or not one is dealing with a secretor or a non secretor because of the relatively high concentrations of saliva and gastric juice necessary to demonstrate the inhibition Consequently, the question as to the specificity of the inhibitory potency of O containing specimens deserves special attention

The best approach to the problem of the existence of an independent substance exhibiting the O property would be through the isolation of such a substance in a chemically pure state In order to achieve this purpose, the isolation of the O substance from gastric juice of human beings belonging to group O was attempted, using the technique described above In this manner the carbohydrate fractions from the gastric juices of several people of group O were isolated and examined for their O potency In regard to the question of specificity, it was important to obtain a satisfactory control preparation The corresponding carbohydrate fractions obtained from gastric juices of people of the non secretor type constitute, without doubt, the most suitable controls Several gastric juices from people of this type were analyzed and their carbohydrate fractions isolated Because of our interest in the isolation of the B substance from gastric juices of human beings belonging to group B as reported in a previous paper, mainly non secretors belonging to group B were used in our investigations so far

The inhibitory activity of these carbohydrate like substances isolated from the gastric juices of human beings of various groups on the isoag glutination of O, A, and B cells was examined in the following way —

Four different carbohydrate-like substances, namely, (1) A specific substance (No 74), (2) B specific substance (Nos 23/24 pooled), (3) O specific substance (No 34), and (4) the carbohydrate fraction isolated from the gastric juice of a non-secretor belonging to group B (No 36) were used in the experiment Decreasing amounts of these substances, volume 0 2 cc , were mixed in part I, with 0 2 cc of undiluted serum of group B, in part II, with 0 2 cc of 1 2 diluted serum of group A, in part III, with 0 2 cc of anti-O beef serum 11 diluted 1 4 Relatively strong serum dilutions were used in this experiment in order to obtain clear-cut results rather than to determine the end point of inhibitory power of the group specific substances under investigation The mixtures were allowed to stand for 15 minutes at room temperature Then 0 2 cc of a 1 per cent suspension of human red blood cells was added as follows to part I, cells belonging to group A, to part II, cells belonging to group B, to part III, cells belonging to group O After standing at room temperature for another 15 minutes, the tubes were centrifuged The readings obtained are given in Table IV

The following conclusions can be drawn from the experiment

1 Only the B substance inhibits agglutination of B cells by serum of group A

2 Only the A substance inhibits agglutination of A cells by serum of group B [1]

3 All three substances, A, B, and O, on the other hand, inhibit agglutination of O cells by the anti-O beef serum

4 The carbohydrate fraction isolated from the gastric juice of a non-secretor belonging to group B lacks any inhibitory potency toward any of the three group specific sera

The results of these experiments indicate that it is possible to isolate a carbohydrate-like substance exhibiting O potency from the gastric juice of human beings belonging to group O Because the results of the O agglutination are usually not as clear-cut as the results of the agglutination of A and B cells by their respective isoagglutinins, the specificity of the inhibition of O agglutination might be questioned However, the failure of the carbohydrate fraction isolated from the gastric juice of a non-secretor to show O potency serves as a negative control and points to the fact that the O potency as exhibited by the respective isolated carbohydrate-like fractions is based upon the presence of an actual substance The gastric juice of the non-secretor apparently contains a carbohydrate-like substance quantitatively and chemically similar to that isolated from the gastric juice of a secretor However, the carbohydrate-like substance isolated from the gastric juice of the non-secretor differs from that of a secretor in regard to its serologic activity That is the reason why it constitutes a most suitable and nec-

[1] Some B preparations give a slight cross reaction with the A preparations and *vice versa* This cross reaction needs further investigation

TABLE IV

Agglutination of Human Red Blood Cells Belonging to Blood Groups O A, and B Respectively by Means of Group Specific Serum Treated with Carbohydrate Like Substances Isolated from Human Gastric Juice

Dilutions of substances 1 1000	A substance	B substance	O substance	Substance from non-secretor Group B

Part I

Agglutination of Human Cells Belonging to Group A by Serum of Group B Treated with the Group Specific Substances

(1) Undiluted	−	++	++++	++++
(2) 1 3	−	++++	++++	++++
(3) 1 9	−	++++	++++	++++
(4) 1 27	−	++++	++++	++++
(5) 1 81	−	++++	++++	++++
(6) 1 240	±	++++	++++	++++
(7) 1 720	++	++++	++++	++++
(8) 1 2200	+++	++++	++++	++++
(9) 1 6600	++++	++++	++++	++++
(10) 0	++++	++++	++++	++++

Part II

Agglutination of Human Cells Belonging to Group B by Serum of Group A Treated with the Group Specific Substances

(1) Undiluted	++++	−	+++	+++
(2) 1 3	++++	−	+++	+++
(3) 1 9	++++	−	++++	+++
(4) 1 27	++++	−	++++	+++
(5) 1 81	++++	−	++++	+++
(6) 1 240	++++	±	++++	++++
(7) 1 720	++++	++	++++	++++
(8) 1 2200	++++	+++	++++	++++
(9) 1 6600	++++	+++	++++	++++
(10) 0	++++	++++	++++	++++

Part III

Agglutination of Human Cells Belonging to Group O by Anti-O Beef Serum Treated with the Group Specific Substances

(1) Undiluted	−	−	−	+++
(2) 1 3	−	−	−	+++
(3) 1 9	−	−	−	+++
(4) 1 27	−	+	−	+++
(5) 1 81	+	+	+	+++
(6) 1 240	+	++	+	+++
(7) 1 720	++	+++	++	+++
(8) 1 2200	++	+++	++	+++
(9) 1 6600	+++	+++	+++	+++
(10) 0	+++	+++	+++	+++

essary negative control in experiments demonstrating the activity of the O substance

So far about 40 specimens of gastric juice have been examined and their carbohydrate fractions isolated In some instances only a few milligrams of the isolated substances were obtained, sufficient to carry out a few tests Table V shows the inhibitory potency toward the O agglutination of six different carbohydrate-like substances isolated from human gastric juices of persons of various blood groups

TABLE V

Agglutination of Human Red Blood Cells Belonging to Group O by Absorbed Beef Serum after Treatment of the Latter with Various Carbohydrate Fractions Isolated from Human Gastric Juice

Dilutions of carbohydrate fractions 1 1000	Carbohydrate fractions diluted 1 1000 belonging to groups					
	(*a*) O	(*b*) O	(*c*) A	(*d*) AB	(*e*) Inert B	(*f*) Inert B
(1) 1 3	−	−	−	−	++	+
(2) 1 6	−	−	−	−	++	+
(3) 1 12	−	−	−	−	+++	++
(4) 1 24	−	−	−	−	+++	++
(5) 1 48	−	−	−	−	+++	++
(6) 1 96	−	−	−	−	+++	+++
(7) 1 190	±	−	+	±	+++	+++
(8) 1 380	+	±	+	+	+++	+++
(9) 1 760	+	+	+	+	+++	+++
(10) 1 1520	++	+	+	+	+++	+++
(11) 1 3040	++	++	++	++	+++	+++
(12) 0	+++	+++	+++	+++	+++	+++

Decreasing amounts of the carbohydrate-like substances diluted 1 1000, volume 0 2 cc , were mixed with 0 1 cc of an absorbed normal beef serum 7 diluted 1 4 After being kept for 4 hours at ice box temperature,[2] 0 1 cc of human red blood cells belonging to group O were added The tubes were kept for 15 minutes at room temperature and then centrifuged The resulting agglutination shown in Table V was obtained after the tubes had been shaken up, this time rather thoroughly The carbohydrate fractions examined in the experiment were as follows (*a*) O substance (No 34), (*b*) substance from non-secretor belonging to group B (No 36), (*c*) O substance (No 45) (*d*) substance *from non secretor belonging to group* B (No 46), (*e*) AB substance (No 47), (*f*) A substance (No 49)

The inhibitory activity of the group specific substances toward the agglutination of human cells belonging to group O by anti-O beef serum is evident On the other hand, the carbohydrate-like substances isolated

[2] In later experiments it proved to be unnecessary to keep the tubes at ice box temperature for 4 hours Instead, they were kept for 15 to 30 minutes at room temperature

from the gastric juice of non secretors belonging to group B do not, or only slightly, inhibit the agglutination of O cells by anti O beef serum It is noteworthy that the carbohydrate like substance isolated from the gastric juice of a patient of group AB (specimen 47) exhibits definite O potency When examined for A and B activity, it proved to be of marked and approximately equal potency for both In a second AB case (specimen 75) the carbohydrate like substance isolated from the gastric juice behaved similarly to the first one However, in this case the inhibition of the O agglutination, although definite, appeared to be somewhat weaker

DISCUSSION

The agglutination of human red blood cells belonging to group O by normal animal sera as well as by certain immune sera has been observed and confirmed by several investigators The problem arises whether or not there is a group specific substance O chemically and serologically comparable to the group specific substances A and B, or whether the O factor is something different and does not constitute an independent group specific substance as such Schiff and his coworkers maintained the conception of an O substance analogous to the A and B substances There are, however, several differences between the A and B agglutination on the one hand and the O agglutination on the other hand For instance, the isoagglutination of human red blood cells of groups A and B by their respective isoagglutinins, provided potent sera are used, is usually stronger and more specific than the agglutination of O cells by anti O beef serum The most striking difference becomes evident when the inhibitory potency of secretions containing the O factor is compared quantitatively with secretions containing the A and the B factors Saliva and gastric juices of human beings belonging to the groups A and B may inhibit the isoagglutination of the respective blood cells in dilutions up to 1 10,000 or more On the other hand, the same secretions of human beings belonging to group O inhibit the O agglutination in dilutions up to one in several hundred, or sometimes less Because of the relatively high concentration of material necessary to demonstrate the O effect in the cases of saliva and gastric juice, which are very viscous, the interference of non specific factors has to be kept in mind Schiff (25) in 1934 thought that it was possible to overcome this difficulty by using anti-O agglutinins found in the serum of a goat immunized by Eisler in Vienna with Shiga dysentery bacilli When this immune serum was used as an anti O reagent instead of the usual normal beef serum, dilutions up to 1 10,000 and more of saliva and gastric juice belonging to group O proved to be active in the inhibition of the O agglutination It is obvious,

FATAL INFECTION OF IRRADIATED WHITE MICE WITH EUROPEAN TYPHUS BY THE INTRA ABDOMINAL ROUTE

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PLATE 33

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The study of classical European typhus in the laboratory has long been handicapped by the lack of suitable experimental animals The guinea pig, which until very recently has been the only convenient species available, usually shows no evidence of infection other than a mild febrile period lasting a few days To prove that this fever is the result of infection with European typhus, it must be shown that typical brain lesions are present, that bacteria cannot be grown from the blood or tissues of the guinea pig, and that resistance to known homologous passage strains has developed after convalescence Furthermore, since 2 to 4 per cent of normal guinea pigs fail to exhibit fever following inoculation of fully virulent material, the inclusion of several animals for each test is essential The interpretation of febrile episodes in guinea pigs without resort to the criteria just noted is hazardous, and it follows, therefore, that rigidly controlled experiments are laborious and time-consuming Demonstration of rickettsiae by tissue culture method or egg inoculation, though very helpful, does not shorten materially the time required to determine the presence or absence of the specific disease

In normal mice, European typhus exists as an inapparent infection in which the virus disappears after three passages, when the method of passing brain to the abdominal cavity is employed (1) Inoculation of the murine variety of rickettsiae, on the other hand according to Wohlrab (2) leads to a fatal outcome in 60 per cent of his strain of mice, whether the inoculum is given intraperitoneally or intranasally Castaneda has described a lethal pneumonitis in mice and rats resulting from the intranasal inoculation of the murine strain (3)

Recently Durand and Sparrow (4), using lice heavily infected with the

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rickettsiae of European typhus, inoculated mice intranasally and were able to pass the infection thus established in series The mortality was high among their animals The mouse obviously possesses advantages in many ways over the guinea pig for the investigation of certain problems, but the intranasal route is dangerous, a fact attested by the occasional human infections in laboratories where this procedure is employed (5) It is especially to be avoided if unvaccinated persons have access to the rooms containing infected animals Since the data presented by Durand and Sparrow (4) show that only a heavy inoculum is effective and since x-radiation increases the susceptibility of rats to murine typhus (6), it seemed probable that, provided large inocula were employed, the intra-abdominal route might prove successful in irradiated mice with European typhus This report describes experiments which were undertaken with these considerations in mind

Methods

X-Radiation —A metal container 3 cm deep and 20 cm in diameter, having a flange about the edge, was fitted with a wire screen (2 or 3 meshes to the inch) which, as a cover, could be clamped tightly to the flange Twenty mice can be accommodated with ease in a container of this sort In it they cannot change appreciably either their distance from the target of the x-ray tube by standing up, or their exposure to the rays by crawling on top of one another Uniform irradiation of each animal is thus obtained The x-ray machine was operated at 200 kilovolts, 8 milliamperes, 0 5 mm copper filter, 30 cm distance Under these conditions an exposure of 7 minutes and 30 seconds represents 450 Roentgen units Data relating to the determination of the optimum interval between the time of irradiation and injection and to the effect of irradiation on non-infected mice will be presented after a description has been given of the basic observations concerning the nature of the infection in irradiated animals

Typhus Strain —The Breinl strain of European typhus obtained from the National Institute of Health in Washington, D C , through the courtesy of Dr R E Dyer, in February, 1940, was isolated in tissue culture (7) and maintained for 5 months by serial passages in the yolk sac of developing chick embryos (8) or on agar tissue cultures until the inception of the experiments described below

Suspensions for injection of mice were prepared from infected chick embryonic tissue incubated at 37°C for 7 days on beef serum-Tyrode agar in Kolle flasks Only those flasks were used which showed many rickettsiae on direct smear The tissue from each Kolle flask was washed down with buffered saline (pH 7 0) and ground to a fine suspension in a special 50 cc centrifuge tube fitted with a pestle In one experiment the tissue from one Kolle flask was diluted with 18 cc , in another with 30 cc The supernatant fluid obtained after the "Griffith tube" had been standing for a few minutes was injected into the mice The suspension was not centrifuged

Establishment of Rickettsial Infection in Irradiated Mice

Injection intra-abdominally of 1 cc of a tissue culture suspension of rickettsiae produced no evidence of sickness in sixteen normal mice This

was confirmed by a second experiment Rickettsiae could not be demonstrated in smears of fluid obtained by aspiration from the peritoneal cavities of such non-irradiated mice 2 to 10 days after injection No titration of the infectivity of the inoculum for guinea pigs was carried out Mice weighing from 10 to 20 gm given 450 to 600 Roentgen units and injected with 1 cc of the inoculum which had been shown to produce no recognizable disease in normal mice, either died or presented evidence of illness (anorexia, ruffled fur, conjunctivitis) Numerous rickettsiae (demonstrated by Macchiavello stain) were visible inside the cells of the peritoneal exudate and in scrapings of the peritoneal surface from the mice which died, as well as in the mouse sacrificed for passage Tissue culture suspensions were inoculated into a total of twenty four irradiated mice, two survived, one was sacrificed in a moribund condition for passage, and twenty-one died

Serial Passage in Irradiated Mice—From one set of irradiated mice infected with material from tissue cultures, three series of transfers in mice were made In two of them material for transfer was taken after the death of the animals, and bacterial infection occurred The third remained free of bacteria and has been carried through twenty two passages by successive intra abdominal inoculation The material for passage was obtained either by bleeding from the heart just before death, or by sacrificing a moribund mouse and washing out the peritoneal cavity with 5 to 10 cc of saline, nutrient broth, or phosphate buffer at pH 7 0 At present broth is employed as routine In other experiments successful transfers were made not only with blood and peritoneal washings but also with brain, lung, pleural exudate, and spleen Rickettsiae were demonstrated by Macchiavello stain in the peritoneal exudate and also in blood, suprarenal gland, tunica vaginalis, kidney, spleen, liver, and pleural fluid, but not in brain Occasionally the lungs of a moribund mouse exhibited hemorrhagic areas varying in size from 1 to 5 or 6 mm in diameter On direct smear rickettsiae could be found in such lesions, chiefly inside the phagocytic cells

Fig 1 shows the appearance of the peritoneal smear on the third and eleventh mouse passages In the latter the rickettsiae are smaller, and many more are present both inside and outside the cells

In a titration of the infectivity of blood after sixteen passages, in one small experiment 0 00017 cc of heart's blood (diluted in nutrient broth) was given intra abdominally to irradiated mice They died 10 to 12 days later, and a few rickettsiae were demonstrable in peritoneal smears at death Brain was found to be infectious only in a dilution of one to five hundred Further titration experiments after the strain has been passed through several more generations will be needed before conclusions can be drawn as to the infectivity of blood and other tissues It is clear, however, that

blood from one moribund mouse was sufficient to infect fatally a considerable number of irradiated mice In routine passage experiments with mice given 450 R, the mortality following injection of 0 5 cc to 1 0 cc of peritoneal washing was regularly 95 to 100 per cent in 4 to 9 days It is especially important to select a moribund animal for passage rather than to use one which has been dead even for a few hours Bacterial contamination in the latter instance was not an infrequent finding in our experiments

Results Obtained in Normal Mice with Passage Material from Irradiated Mice —Normal mice failed to show any evidence of infection when injected with passage material from irradiated infected mice Eighty-six animals were inoculated with peritoneal washing, blood, or brain obtained from the first to the fifteenth passages, inclusive, and from the eighteenth passage Attempts were made to infect very young mice (1 day to 3 weeks old) on five occasions In two trials rickettsiae were demonstrable at death of the baby mice, but three additional experiments resulted entirely in failure It can be stated at present that as a result of eighteen passages in irradiated mice the strain had not acquired virulence for non-radiated mice with the possible exception of newborn animals

Behavior of the Mouse Passage Strain in Guinea Pigs —To show whether the organisms observed in the irradiated mice after serial passage were identical with the typhus rickettsiae originally introduced, guinea pigs were inoculated intra-abdominally with material from the first, second, third, fourth, and eleventh mouse passages, allowed to recover, and then tested for immunity to the Breinl strain which had been maintained continuously in the guinea pig The daily temperatures of guinea pigs after injection with mouse passage material are recorded in Table I*a* and Table II*b* (Nos 7-84, 7-29, 7-31, 7-89) Table I*b* represents the temperatures of some of those animals following the test dose with the Breinl strain (0 1 gm of guinea pig brain removed on the 3rd or 4th day of fever), along with the temperatures of ten normal guinea pigs injected with the same quantity of the same suspension of brain It should be stated that for the purpose of an experiment unrelated to this work forty-two normal guinea pigs were injected with portions of the same pooled inoculum The ten described here in detail are representative Of the forty-two normals, forty showed fever curves characteristic of European typhus Temperatures over 104°F (40°C) are set in bold-faced type and are taken to indicate fever in our stock of guinea pigs It is evident from Table I*a* and *b* that the guinea pigs given material from the first few passages in irradiated mice exhibited an irregular, atypical febrile response, but all were immune to a dose of infective guinea pig blood and brain which caused fever in forty of forty-two normal controls

Blood was taken on the 3rd day of fever from one of the animals infected with mouse material of the fourth passage (guinea pig 30) for culture and injection intra abdominally into a normal guinea pig (No 38) No bacteria

TABLE I*a*

Temperatures of Guinea Pigs after Injection of Brain Pleural Fluid or Peritoneal Washings of Irradiated Infected Mice First to Fourth Passages Inclusive

(Temperatures in degrees above 100°F)

Guinea pig No	Inoculum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
9	Per wash mouse 1st gen	3 0	4 1	2 2	2 3	3 2	3 4	3 4	4 1	3 5	3 6	2 1	2 0	1 8	2 6	4 6	2 2	1 8	2 1	1 8	2 2	2 4	
13	Brain mouse 1st gen	4 0	3 0	1 7	4 2	5 6	4 5	3 9	2 7	2 2	1 8	2 4	3 4	4 5	4 7	3 9	4 6	5 0	5 6	4 2	3 3	2 8	
12	Pleural fl mouse 1st gen	3 8	3 2	3 0	2 8	2 9	3 1	3 0	2 4	1 9	2 2	3 2	4 6	4 7	4 5	4 0	3 0	4 2	4 1	3 5	3 2	2 0	
7 99	Per wash mouse 2nd gen		3 0	1 7	2 9	3 9	4 5	4 8	5 4	4 3	4 6	4 7	5 0	3 9	4 7	2 4	2 5	2 0	2 4	2 7	3 1	2 6	
92	Brain mouse 1st gen	2 8	4 2	2 7	3 8	3 7	3 8	3 2	3 0	4 9	4 1	3 6	3 8	2 3	3 0	2 8	3 0	3 6	2 4	3 5	2 4	2 6	
22	Per wash mouse3rdgen	3 5	2 5	1 0	2 5	4 1	4 4	4 7	4 6	3 9	5 2	4 6	4 5	3 7	4 4	2 2	2 0	2 0					
30	4th	3 6	3 5	4 5	5 6	5 9	4 9	5 0	2 3	4 3	4 8	4 7	4 5	4 0	3 2	2 0	1 8	2 6	2 7	2 3			
16	4th	2 9	3 5	3 8	3 9	4 4	3 9	5 2	5 3	5 4	4 0	3 8	3 4	3 0	2 4	2 4	2 5	2 1	2 6				
38	Blood pig 30	2 0	1 3	1 8	4 9	2 4	2 2	3 8	3 8	4 6	4 6	5 6	4 7	5 3	4 8	4 7	4 0	3 0	2 9	2 5	2 7		

TABLE I*b*

Temperatures of Guinea Pigs Recovered from Infection with Mouse Material Given Subsequent Inoculum of Brein Passage

(Temperatures in degrees above 100 F)

9	Reco ered from infect on		2 7	1 7	2 3	2 7	1 8	2 0	2 0	1 9	2 0	1 4	2 6	1 8	1 8	1 9	1 1	1 2	1 8	2 3									
13	with mouse material		1 8	1 3	2 6	3 2	2 0	2 6	2 4	1 7	2 3	2 2	1 8	1 9	2 2	2 8	2 4	3 4	2 9	2 2									
12			1 9	2 2	2 1	2 1	2 0	2 9	2 0	1 6	2 4	1 7	2 0	2 4	1 8	1 8	1 9	1 9	1 4										
7 99			2 5	3 5	2 7	3 2	3 0	2 8	2 6	2 9	2 4	2 0	2 8	2 3	3 0	2 2	2 2	1 8	2 4	2 0									
92			3 0	2 1	2 3	3 2	2 7	2 9	2 2		2 3	2 2	2 1	2 7	3 7	2 8	4 0	2 7	3 2	2 7									
22			2 6	2 8	2 9	3 5	3 1	2 2	2 6	2 0	2 2	1 6	2 8	2 0	2 6	2 5	1 8	2 0	1 7	2 2									
30			2 8	2 2	2 5	2 6	1 8	2 0	2 8	2 6	1 8	1 6	2 7	2 0	2 8	2 5	1 8	2 2	1 9	2 0									
16			2 8	1 7	2 5	3 4	2 4	2 2	2 0	2 0	2 3	2 0	2 6	1 9	2 3	2 1	2 3	2 6	2 6	2 5									
38			2 6	3 0	2 5	2 6	2 2	3 0	2 6	3 0	2 8	3 0	3 6	2 4	2 1	2 6	2 8	1 7	2 8	2 6	2 4								
7-64	Normal controls	3 0	3 8	3 2	2 7	2 7	2 8	2 2	2 2	3 0	5 3	4 7	5 0	5 2	5 6	3 9	4 1	3 6	2 3	2 2									
7 53		3 0	3 6	3 3	3 7	2 8	2 7	2 7	3 3	3 5	2 8	5 4	4 7	5 8	5 6	5 6	5 3	4 8	4 0	3 2	4 0	2 5	2 1						
7-80		3 5	2 8	3 1	2 9	2 9	2 6		7 3	0 3	4 3	6 5	4 4	7 4	5 4	8 4	9 4	9 4	5 3	0 2	8 2	5						
7-56		3 3	2 8	3 0	2 8	3 0	2 6	2 8	3 2	3 6	3 6	3 8	4 6	4 6	5 5	5 4	4 5	3 5	3 4	2 8									
7-66		3 5	1 2	2 0	3 4	3 4	3 5	3 0	3 3	3 5	4 8	5 0	4 3	4 5	4 8	4 7	4 2	3 5	2 8	2 4									
7 50		3 8	2 6		7 2	7 2	2 3	3 2	7 2	9 3	7 3	6 3	7 4	7 5	2 5	2 5	4 5	0 4	8 4	2 4	3 3	3 3	6 2	9					
7-61		3 5	3 2	2 5	3 0	2 9	2 9	1 9	3 6	3 5	4 7	3 3	8 5	8 5	1 5	2 5	0 4	1 3	8 3	4 3									
7 55		2 3	3 6	3 2	2 6	3 3	3 2	3 5	2 5	2 7	4 0	4 6	4 5	4 5	4 8	4 8	4 0	3 8	3 0	2 5									
7-38		3 8	2 5	2 5	2 9	2 1	3 0	3 0	2 0	2 5	3 2	4 6	4 6	4 8	4 8	4 5	4 3	3 3	3 2	2 8	2 8								
7-45		2 8	3 2	3 3	3 2	2 8	3 0	2 5	3 1	3 0	3 3	4 5	4 0	3 8	3 8	3 6	2 6	3 0	2 8										

were found in the blood culture (3 cc blood, 50 cc broth, 2 weeks' incubation) The temperatures of guinea pig 38 are recorded in Table I*a* and are typical of European typhus in the guinea pig (In a later experiment, three or four guinea pig passages sometimes were made before the temperature curve became consistent and typical of European typhus) On subse

blood from one moribund mouse was sufficient to infect fatally a considerable number of irradiated mice In routine passage experiments with mice given 450 R, the mortality following injection of 0 5 cc to 1 0 cc of peritoneal washing was regularly 95 to 100 per cent in 4 to 9 days It is especially important to select a moribund animal for passage rather than to use one which has been dead even for a few hours Bacterial contamination in the latter instance was not an infrequent finding in our experiments

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Behavior of the Mouse Passage Strain in Guinea Pigs —To show whether the organisms observed in the irradiated mice after serial passage were identical with the typhus rickettsiae originally introduced, guinea pigs were inoculated intra-abdominally with material from the first, second, third, fourth, and eleventh mouse passages, allowed to recover, and then tested for immunity to the Breinl strain which had been maintained continuously in the guinea pig The daily temperatures of guinea pigs after injection with mouse passage material are recorded in Table I*a* and Table II*b* (Nos 7-84, 7-29, 7-31, 7-89) Table I*b* represents the temperatures of some of those animals following the test dose with the Breinl strain (0 1 gm of guinea pig brain removed on the 3rd or 4th day of fever), along with the temperatures of ten normal guinea pigs injected with the same quantity of the same suspension of brain It should be stated that for the purpose of an experiment unrelated to this work forty-two normal guinea pigs were injected with portions of the same pooled inoculum The ten described here in detail are representative Of the forty-two normals, forty showed fever curves characteristic of European typhus Temperatures over 104°F (40°C) are set in bold-faced type and are taken to indicate fever in our stock of guinea pigs It is evident from Table I*a* and *b* that the guinea pigs given material from the first few passages in irradiated mice exhibited an irregular, atypical febrile response, but all were immune to a dose of infective guinea pig blood and brain which caused fever in forty of forty-two normal controls

viously uninfected pigs (No 7 89) was sacrificed on the 10th day to initiate a new guinea pig strain which will be referred to as the M XI strain since it derives from the eleventh mouse passage It was maintained by routine

TABLE III*a*

Temperatures of Guinea Pigs Given Routine Inoculum of Breinl Guinea Pig Brain

(Temperatures in degrees above 100 F)

Guinea pig No	Previous infection	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
7-09	None	3 2		2 8	3 0	2 9	3 8	3 9	4 6	4 3	4 7	4 6	5 0	4 6	4 2	3.5	3 0	4 0	3 3	2 9	3 4	
7 20		2 0	2 0	1 0	2 8	1 6	4 6	4 8	4 6	5 5	5 1	5 0	5 0	4 8	3 5	2 3	2 9	1 8				
7-33		3 6		2 7	2 7	2 6	4 7	3 9	4 0	4 2	4 1	5 1	5 4	4 3	4 7	3 7	3 5	3 0				
7-34		2 3	2 4	3 0	2 5	3 3	3 7	4 7	5 5	4 0	4 5	4 5	4 4	4 3	3 3	3 4	2 5					
7-37		3 8		2 3	3 8	3 5	4 3	4 0	4 7	4 0	4 6	4 6	5 4	4 7	5 2	3 9	3 2	3 0				
7-62		3 8	1 5	2 8	2 5	3 0	2 5	3 5	2 6	3 0	3 8	4 0	4 5	5 2	4 7	5 7	4 5	4 0	4 6	2 5	2 4	2 4
7-69		3 3		1 7	3 5	2 1	2 6	2 9	2 6	2 8	3 3	2 7	5 1	4 4	4 5	4 0	4 5	4 5	4 8	4 7	3 6	2 6
7-48		2 5		1 5	5 5	3 1	3 8	4 0	3 2	4 3	4 6	4 1	5 4	5 4	4 8	3 8	4 9	3 8	3 0	3 0		
7 72		2 8		2 0	2 1	2 6	2 5	4 2	4 5	4 0	4 7	4 6	4 7	4 6	4 2	3 0	2 4	2 0				
7 75		3 5		3 2	2 8	2 7	3 0	3 0	4 7	4 0	4 5	4 0	4 8	4 6	4 6	3 8	3 1	2 6				
7-81		2 4		2 6	2 5	3 6	3 7	3 2	5 0	3 4	4 5	4 0	4 8	4 6	4 0	4 1	3 3	2 6	2 2			
7 94		3 0		2 2	3 1	4 0	3 8	3 8	4 4	3 6	5 0	5 0	4 4	4 4	3 5	3 4	2 4					

TABLE III*b*

Temperatures of Recovered Breinl Guinea Pigs and Normal Controls Injected with Brain of M XI Strain 6th Generation

(Temperatures in degrees above 100 F)

7-09	B eml passage	2 8	2 7	3 0	2 8	3 3	2 8	3 3	2 7	3 5	1 9	1 8	3 0	2 5	2 6	3 4	2 8	2 8	2 7	2 7	2 7	2 9
7 20		3 5	3 0	3 5	3 0	2 3	3 0	3 3	9	2 8	2 6	2 8	2 6	2 5	2 3	3 3	3 5	3 1	3 0	2 7	2 1	3 0
7-33		2 6	2 8	2 9	3 0	2 8	2 5	2 7	2 3	3 3	2 7	2 6	2 6	2 5	2 4	3 3	2 8	2 9	2 7	2 1	2 1	2 6
7-34		2 7	2 9	2 7	2 6	3 6	3 1	3 5	2 8	3 3	2 9	3 1	3 7	3 0	2 5	3 5	3 5	2 7	2 5	3 4	2 9	2 9
7-37		2 6	3 3	2 5	3 3	2 5	3 5	2 7	2 1	3 0	2 3	2 7	2 7	2 6	1 8	3 7	3 2	2 5	2 0	2 3	1 8	3 5
7-62		3 2	2 8	2 9	3 6	4 3	3 6	2 8	2 7	2 8	2 8	2 8	3 5	2 8	2 6	3 0	3 9	2 5	2 8	3 3	2 5	2 5
7-69		2 7	2 9	2 8	3 5	3 4	2 7	3 2	2 7	3 0	2 6	2 5	3 3	3 0	2 3	3 1	2 8	2 4	3 1	3 0	3 0	3 0
7-48		2 8	2 4	3 6	2 7	3 6	4 0	3 5	2 8	3 6	3 3	3 4	3 5	3 6	2 9	4 2	2 5	2 6	3 0	5	3 0	3.2
7 72		3 1	3 1	3 5	3 2	3 1	2 0	3 1	3 0	3 1	2 3	3 1	2 9	3 1	2 3	3 2	3 3	3 1	2 7	3 0	2 7	3 0
7 75		2 8	2 9	3 5	3 1	3 5	3 5	3 3	2 9	3 5	2 7	2 5	3 0	2 2	2 5	3 5	4	2 4	2 0	2 8	1 8	2 6
7-81		2 9	3 5	3 5	2 8	3 0	2 1	3 0	2 7	3 5	3 4	2 7	3 0	3 2	2 1	3 3	3 1	3 0	2 8	2 8	3 1	3 3
7-94		2 9	2 6	3 0	3 1	3 5	3 9	4 1	4 1	3 5	2 9	3 1	3 3	2 9	2 5	3 2	3 2	2 9	2 9	3 0	3 0	3 1
17-02	None	3 5	4 0	3 1	2 9	4 8	4 1	3 9	3 9	4 5	3 5	4 5	4 8	4 8	4 8	4 9	4 1	4 1	3 7	3 5		
17-03		2 2	3 7	4 4	4 4	3 4	3 7	3 9	4 1	4 5	5 0	4 8	4 6	5 8	5 5	5 0	4 8	4 4	3 7	3 3	3 4	
17 10		2 8	2 9	2 4	2 5	3 4	4 5	3 6	4 6	4 4	4 7	5 8										
17 11		3 5	4 1	3 6	3 0	3 7	4 5	4 6	4 9	5 3	4 9	5 9	6 0	5 9	5 2	4 9	4 6	3 9	3 5	3 2		
17 16		2 5	2 0	2 0	1 9	3 1	3 3	3 5	4 0	4 6	4 6	4 9	5 1	5 8	5 6	6.2	6 4	5 8	2 0	†		

* Sacrificed for passage

† Died of intercurrent bacterial infection

brain passage through seven generations of guinea pigs Twelve recovered Breinl guinea pigs, whose original infection is recorded in Table III*a*, were given 0 1 gm of the brain and 0 2 cc. defibrinated blood of the fifth guinea pig passage of M XI, six normal control animals received identical

quent immunity tests with the guinea pig passage strain, the second generation pig 38 showed no fever (Table I*b*, No 38) The controls for guinea pig 38 are not included in Table I*b*, twenty normal guinea pigs received 0 1 gm of 10 per cent brain suspension of Breinl passage animals and nineteen of the twenty showed characteristic temperature curves

TABLE II *a*

Temperatures of Normal Guinea Pigs Given Routine Inoculum of Breinl Guinea Pig Brain

(Temperatures in degrees above 100°F)

Guinea pig No	Previous infection	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
7 04	None	4 2	2 6	3 2	2 9	3 7	3 3	4 5	5 0	4 7	4 7	5 2	5 1	4 5	4 0	3 3	2 7	1 7			
7-16	"	3 6	3 3	3 0	2 7	3 6	3 7	4 6	4 8	3 8	4 6	3 8	4 5	3 8	2 8	3 5	3 4				
7-23	"	4 6	3 0	4 3	2 9	3 8	2 0	4 5	5 2	5 1	5 8	5 0	5 3	5 0	4 6	4 4	4 3	3 7	3 4	3 2	
7-24	"	2 8	2 0	2 6	2 5	2 8	2 3	2 5	2 5	2 7	2 9	5 0	3 6	4 5	4 7	5 0	4 6	3 1	2 7	2 2	
7-46	"	2 6	2 9	2 8	2 8	2 9	2 9	3 5	3 4	4 7	4 0	3 7	5 8	5 7	5 7	5 3	5 8	5 0	4 0	3 2	1 8

TABLE II *b*

Temperatures of Recovered Breinl Guinea Pigs and Normal Controls after Injection of Peritoneal Washing of Irradiated Infected Mice, Eleventh Passage

(Temperatures in degrees above 100 0°F)

7-84	None	4 0	3 7	5 2	4 2	3 9	5 3	5 3	4 7	4 6	4 2	3 6	2 6	2 6		2 2	2 0								
7-29	"	3 5	3 6	5 0	4 6	3 5	5 5	4 2	2 9	3 6	4 0	3 0	1 9	2 2		1 6	1 6								
7-31	"	3 0	2 6	4 5	3 6	3 5	4 0	4 5	4 0	4 0	3 0	3 5	3 4	2 8		2 7	1 9								
7-89	"	3 8	3 6	4 8	5 3	2 5	5 0	5 3	5 0	4 9	*														
7 04	Breinl	2 5	2 8	3 3	2 6	1 7	2 7	2 3	2 5	3 2	2 5	3 3	1 8	2 3	2 4	1 8	1 6								
7-16	passage	4 8	2 0	3 0	1 6	1 8	2 8	2 6	2 0	2 5	2 5	3 7	2 0	2 3	2 6	2 3	1 0								
7-23	" "	3 5	2 8	3 1	2 9	2 0	3 0	2 9	2 9	2 8	3 6	3 2	2 6	2 2	2 8	2 5	2 5								
7-24	" "	4 5	1 7	1 8	2 5	3 0	2 5	2 0	1 5	2 5	2 1	2 1	2 0	2 1	1 7	1 6	1 3								
7-46	" "	3 5	2 6	2 6	2 6	2 3	2 9	2 9	2 6	2 3	3 3	3 2	7 2	3 2	5 2	4 1	4 1	7							

* Sacrificed for passage

To test for complete cross immunity with the Breinl strain, five guinea pigs which had received guinea pig passage material and which had exhibited typical typhus temperature responses (Table *IIa*) were given a rest period of 3 weeks or more and then injected intra-abdominally with 1 cc of mouse peritoneal washing of the eleventh passage At the same time four normal guinea pigs were injected with the same inoculum (Table *IIb*) The normal guinea pigs developed an early fever and two exhibited very slight scrotal swelling on the 4th day, whereas in the recovered Breinl pigs neither of these morbid manifestations was observed One of the pre-

results suggest that an interval of 1 to 3 days between irradiation and infection is more suitable than an interval of only a few hours, in that the animals succumb much more quickly after inoculation These results have been confirmed in another experiment similar to that shown in Table IV

Effect of Irradiation on Mice Not Injected with Infective Material—In these experiments an arbitrary period of observation has been set at 14 days The percentage of uninfected irradiated mice dying within this interval

TABLE V

Effect of Irradiation on Uninfected Control Mice

Date	Dose of x ray	No of mice	No dead end of 14 days	Per cent dead end of 14 days	Average day of death
				per cent	
Oct 17	500 R	5	2	40	9 5
19		6	0	0	—
22		6	1	17	13
26		5	2	40	10
28		6	1	17	12
29	'	6	2	33	12 5
30		6	1	17	14
31		6	0	0	—
Nov 4	'	6	1	17	8
23		6	0	0	—
Dec 17		5	4	80	11
Nov 9	450 R	10	0	0	—
21		5	0	0	—
27		6	0	0	—
30		6	0	0	—
Dec. 4		5	0	0	—
9		6	0	0	—
13		5	0	0	—

is shown in Table V which presents data for 63 uninfected control mice given 500 R units and 43 given 450 R units Of the 500 R group, 23 per cent were dead at the end of 14 days, in the 450 R group all animals survived the entire period The survivors frequently show ruffled fur and may not eat for several days The injection intra abdominally of saline 2 or 3 days after irradiation may possibly lessen the severity of postradiation sickness, but no attempt was made to determine this point accurately The irradiated control mice for the experiments here reported received intra abdominally as routine either 0 5 cc normal saline or 0 5 cc of the peritoneal washing of a previously irradiated but *uninfected* mouse so that the control injection would correspond as closely as possible to that used in the passage

inocula The course of the infection in these animals is found in Table III*b* from which it can be clearly seen that residence of the strain in mice had not altered appreciably its capacity to persist in serial guinea pig passage or led to obvious changes in immunologic properties The absence of scrotal swelling as well as the finding of rickettsiae in the tunica cells during early stage of infection after long search in the M XI strain, and the similarity of the temperature curve to the Breinl strain maintained constantly in guinea pigs indicate that no permanent change in its characteristics had taken place as a consequence of eleven mouse passages

TABLE IV

Effect of Irradiation of Mice at Different Intervals before Injection of Infectious Material

	No mice	Date of x ray (500 R)	Date of infection	Deaths, time after injection	Average day of death	Survival at 14th day
				days		*per cent*
Lot A	4	Oct 28	Oct 31	4, 6, 7, 7	6 0	0 0
Lot B	4	29	31	4, 5, 6, 6	5 2	0 0
Lot C	4	30	31	3, 5, 6, 8	5 5	0 0
Lot D	4	31	31	10, 10, 10, 10	10	0 0
	No mice	Date of x ray (500 R)		Deaths, time after irradiation		Survival at 14th day
				days		*per cent*
Lot A	6	Oct 28	Controls	12		83
Lot B	6	29		12, 13		66
Lot C	6	30		14		83
Lot D	6	31				100

Microscopic sections of brains of first and sixth generation M XI guinea pigs were made, and lesions typical of typhus were demonstrated in each

Optimum Interval between Irradiation and Injection —The effect of varying intervals of time between irradiation and infection is illustrated in Table IV Four lots of mice of approximately the same weight were given 500 R units as follows lot A, Oct 28, lot B, Oct 29, lot C, Oct 30, lot D, Oct 31 An inoculum of peritoneal washing of an irradiated, infected moribund mouse (ninth passage) was prepared Oct 31 and 0 5 cc injected intra-abdominally into four mice of each lot about 5 hours after the irradiation of lot D The remaining six mice of each lot served as controls and received 0 5 cc of saline washing of the peritoneum of an *uninfected,* irradiated mouse In lot A, x-rayed 3 days before injection, death occurred 6 days after infection on the average, lot B, x-rayed 2 days before injection, 5 2 days after infection, lot C, x-rayed the day before injection, 5 5 days after infection, lot D, x-rayed and injected on the same day, 10 days after infection These

intra abdominal route Rickettsiae were abundant and easily demonstrable in the moribund or dead mice

The mortality of irradiated mice infected with passage material (peritoneal washings or blood) was nearly 100 per cent as contrasted to no mortality in the control mice given the same dose of x ray (450 R) and the same volume of fluid intra abdominally (The observation period of control mice was arbitrarily limited to 14 days)

After eighteen passages in irradiated mice no increase in virulence for non irradiated adult mice was detected

After passage in guinea pigs, the rickettsial infection deriving from the mouse passage material was identical with the Breinl strain as judged by fever, cross immunity tests, and brain lesions in sections

We wish to thank the x ray staff of the Huntington Memorial Hospital of Boston for their advice and cooperation

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experiments Under these circumstances organisms other than rickettsiae but which resembled them might have become apparent in our strain of mice (especially if irradiation enhanced the conditions for the appearance of mouse pathogens) Although bacterial contamination of the peritoneal cavity occurred quickly in irradiated mice following death, no organisms which could easily be mistaken for rickettsiae were demonstrated during the course of these experiments

DISCUSSION

In comparison with the intranasal route used by Castaneda (3) with murine typhus and by Durand and Sparrow (4) with European typhus, the intra-abdominal inoculation of irradiated mice provides a method which we believe is safer for the study of European typhus in the laboratory Moreover, the results reported here suggest that it may well be possible to devise convenient and accurate techniques for testing the protective capacity of vaccines and of antisera obtained from experimental animals and convalescent or vaccinated human beings The intra-abdominal route permits a more accurate measure of the quantities of infectious material or protective serum administered than does the intranasal route Titrations of the infectivity of various materials may also prove practicable and inexpensive through the use of the irradiated mouse Experiments with these objectives in mind are now in progress

The high mortality of the infection, its relatively short duration (as opposed to guinea pig experiments), the ease with which rickettsiae may be demonstrated in the dead or dying mice, its simplicity as an experimental method when contrasted with the taking of guinea pig temperatures daily for several weeks, represent distinct advantages of this method

The chief disadvantage of the technique lies in the necessity for employing irradiation Obviously it would be much more satisfactory if the strain of typhus rickettsia could be adapted to normal mice, thereby reducing the hazard of apparent or latent infection with other organisms which the use of the x-ray may increase

In regions where fertile eggs are expensive or cannot be secured, this method may prove practical for the production of vaccine on a moderately large scale Tests for the efficacy of vaccines prepared from peritoneal washings of irradiated mice are now in progress

SUMMARY AND CONCLUSIONS

A fatal infection of irradiated white mice with the Breinl strain of European typhus has been established and passed serially for 22 passages by the

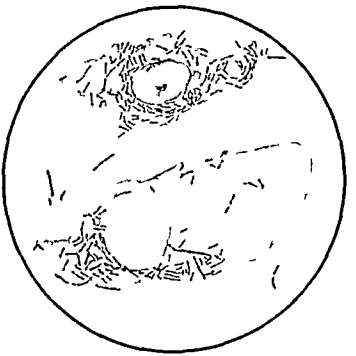

FIG 1*a*

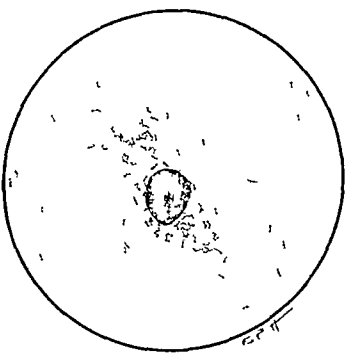

FIG 1*b*

EXPLANATION OF PLATE 33

Fig 1 Camera lucida drawings of the cellular peritoneal exudates of irradiated mice infected with European typhus rickettsiae

(*a*) The third passage in mouse infected with blood

(*b*) The eleventh mouse passage infected with blood

Note the comparative richness of intracellular and extracellular rickettsiae in earlier and later mouse passages Stained by Macchiavello stain × 1300

QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

I A METHOD*†

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Whether complement or alexin be measured in hemolytic, opsonic, bactericidal, or combining units, these units refer only to the smallest volume of guinea pig or other serum producing the effect in question and give no indication of the actual amount of complement involved The present study was undertaken in the hope of filling this gap and providing, in place of these relative and often variable units, an absolute measure of complement in weight units The need for this seemed all the more indicated by the extension of knowledge and the results of theoretical and practical interest that followed substitution of the old relative dilution methods by quantitative, absolute methods of antigen and antibody estimation conforming to the criteria of analytical chemistry (1–3)

It has been known for many years that the titer of added complement or alexin is reduced by antigen antibody precipitation in rabbit antisera, but the actual addition of a complement component to such precipitates has not been shown directly although hemolysis is generally interpreted as mediated by the addition of complement to the red cell Indeed, Muir (4) has defined complement as "that labile substance of normal serum which is taken up by the combination of an antigen and its anti substance (immune body)" Owing to the accuracy with which specifically precipitable nitrogen may be measured, the quantitative precipitin method (5) seemed worthy of trial as a means of estimating any actual uptake of complement It was thought that any difference between the amounts of specifically precipitable nitrogen found in the presence of active complement, on the one hand, and in the presence of inactivated complement on the other, might serve as a measure, in milligrams of nitrogen per milliliter,

*The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital

† A preliminary note was published in *Science* 1940 92, 534

of complement as defined by Muir, or of the combining component or components, or "mid-piece," of a more complex complement Only after considerable experimentation was it realized, however, that specific precipitates should be capable of combining with far more complement, in volume units, than had ordinarily been supposed, and that relatively large volumes of guinea pig serum would be needed if a weight unit for complement were to be established with any degree of accuracy For this reason many earlier experiments are omitted

Methods and Materials

Complement—Sera from a number of large guinea pigs were pooled The lots for Experiments 1 to 5 were neutralized in the cold to about pH 7 (phenol red) by dropwise addition of $\frac{N}{10}$ HCl In other experiments neutralization was either omitted or carried out by means of 0 15 $\frac{N}{10}$ HCl in 0 9 per cent saline Differences between the neutralized and the corresponding untreated samples were scarcely greater than the analytical error

Inactivation by the standard procedure, 30 minutes at 56°C , did not always suffice to reduce to a minimum the amount of nitrogen taken up by specific precipitates from inactivated complement For inactivation in the later experiments the guinea pig serum was rapidly heated in a water bath at 56° until a thermometer in the serum reached 56°, after which heating was continued for 45 to 50 minutes The inactivated serum was chilled and, with the corresponding active serum, was allowed to stand in the cold overnight and centrifuged before use

Sheep Red Cells and Hemolysin—These were obtained from the Wassermann Laboratory of the Presbyterian Hospital[1] and were the usual 5 per cent sheep cell suspension and hemolysin dilution used for the Wassermann test An equal volume of hemolysin dilution containing 2 "units" (estimated in the usual way with excess complement) per 0 1 ml was stirred into the cell suspension As so diluted the cell count varied from about 830,000 to 1,500,000 per c mm ,[2] but this did not appear to affect the hemolytic titer found for the active guinea pig serum even though an equal volume of hemolysin dilution was used in each instance

Hemolytic Titer of Complement—This was estimated while the precipitin nitrogen analyses were being run by addition of varying quantities of 1 50 or 1 100 guinea pig serum to 0 2 ml of the sensitized red cell suspension and incubation in a water bath at 37–39°C for 20 minutes The number of hemolytic units per milliliter of undiluted serum was calculated from the smallest volume giving complete hemolysis Analyses are reported only on pools showing more than 150 per ml of the "units" used

Estimation of Specifically Precipitable Nitrogen—Quantitative determinations were made (5) with proportions of antigen and rabbit antisera so chosen that not quite all of the antibody was precipitated This avoided the formation of disc like precipitates which are difficult to disintegrate and wash thoroughly Since the measurement of a presumably small difference between two quantities of precipitate nitrogen was to be

[1] Through the kindness of Miss Edna Baker, in charge

[2] Counts kindly made by Mrs Katherine E Smith of the Hematological Laboratory, Presbyterian Hospital

attempted analyses were run in triplicate unless otherwise indicated as an additional precaution to ensure the greatest possible accuracy Moreover all supernatants were again centrifuged, as in the quantitative agglutination procedure (6) and on account of the relatively large amounts of guinea pig serum required, all tubes were washed three times with chilled saline instead of twice as is usual for ordinary analyses (5 6) Data given and conclusions drawn by Haurowitz (7) as a result of experiments along the same lines indicate that the technical difficulties involved were underestimated by this worker

The rabbit antisera used were neutralized and inactivated for Experiments 1 to 5 and were of high antibody content so that analyses could be made at dilutions which were not anticomplementary In Experiments 1 to 5 1 0 ml portions of rabbit anti serum dilution were added (each in triplicate) to 5 ml samples of 0 9 per cent saline, 5 0 ml portions of heat inactivated guinea pig serum and 5 0 ml samples of an unheated portion of the same guinea pig serum pool The contents of the tubes were mixed and 1 0 ml of antigen dilution was added to each tube and mixed Blank tubes were also set up with active or inactivated complement to which saline antigen, or antiserum alone was added After 1 hour at room temperature or longer if aggregation did not occur within 1 hour in the active complement series the tubes were centrifuged in the cold[3] and the analyses were completed as described above In the later experiments neutrali zation and inactivation of the rabbit antisera were omitted owing to the high dilutions used

Hemolytic units in the supernatants from the tubes which had contained active com plement were computed from the largest non anticomplementary volume failing to show appreciable hemolysis, and were therefore actually much less than the number indicated except in Experiments 2 and 5, in which complete hemolysis was actually obtained at the level given Washings from the precipitin tubes which had contained active comple ment were also tested and found free from complement, showing that dissociation did not take place during the washing procedure This was also noted by earlier workers

EXPERIMENTAL

Experiment 1.—Temperature 23 C 45 ml of neutralized, centrifuged guinea pig serum (C')[4] used, of which one half was inactivated (iC) Anti Pn[4] III rabbit serum 6.06_{25} 7 0 mg antibody N per ml diluted with 6 volumes saline neutralized and in activated S III in saline 0 04 mg per ml C ' titer 250 hemolytic units per ml

---

[3] Using a refrigerated centrifuge manufactured by the International Equipment Company Boston

[4] Owing to the frequent use of the symbol C to denote bacterial somatic specific poly saccharides it is proposed in agreement with Dr Ecker and Dr Pillemer to use the symbol C for complement and iC for inactivated complement So called mid piece would then be C 1 to denote first component end piece = C 2 and third and fourth components C 3 and C'4 In the present papers the nitrogen added to specific pre cipitates is designated C 1 N as it is generally stated that only mid piece acts as com bining component in such instances However this may be an oversimplification (com munication from Dr Ecker and Dr Pillemer)

Pn is used for pneumococcus S with the appropriate numeral for type-specific poly saccharide of Pn

No of tubes	1	1	2	1	1	3	3	3
C′, *ml*	4 0	4 0						5 0
1C′, *ml*				3 0	3 0		5 0	
Serum dilution, *ml*	0 8		1 0	0 6		1 0	1 0	1 0
S III dilution, *ml*		0 8			0 6	1 0	1 0	1 0
N precipitated, *mg*	0 012	0 016	0	0 018	0 020	0 584 0 594 0 586	0 634 0 624 0 636	0 740 0 738 0 736
Mean	0 014			0 019		0 588	0 632	0 738
Subtraction of blank						0	0 032*	0 018†
Specific N pptd , *mg*						0 588	0 600	0 720
Subtraction of 1C′ series value								0 600
C′1 N pptd , *mg*								0 12

Hemolytic units left in C′ series supernatants, <10 per 5 ml C′ taken
* 0 019 × 5/3
† 0 014 × 5/4

Experiment 2 —Temperature, 23–25°C Neutralized, centrifuged guinea pig serum first filtered through gradocol membranes, 700 mμ average pore diameter[5] 52 ml filtrate, 250 hemolytic units per ml , 22 ml C′ inactivated Same rabbit serum diluted with 9 volumes saline, inactivated, filtered as above, neutralized S III in saline, 0 028 mg per ml

No of tubes	2	1	1	2	3	3	3	1
C′, *ml*	5 0*	5 0					5 0	5 0
1C′, *ml*			5 0			5 0		
Serum dilution, *ml*	1 0*		1 0	1 0	1 0	1 0	1 0	0 5
S III dilution, *ml*		0 5			1 0	1 0	1 0	0 5
Saline, *ml*	1*		1	4	4			
N pptd , *mg*	0 006	0 012†	0 016	0	0 392 0 388 0 386	0 422 0 416 0 426	0 570 0 574 0 578	0 274
Mean	0 009		0 016		0 389	0 421	0 574	0 274
Subtraction of blank					0	0 016	0 009	0
Specific N pptd , *mg*					0 389	0 405	0 565	0 274
Subtraction of 1C′ series value							0 405	0 203‡
C′ 1 N pptd , *mg*							0 16	0 07

Hemolytic units left in C′ series supernatants, 40
" " " " C′, 0 5 S III, 0 5 serum supernatants, 200

* 0 9 of these quantities actually used in the second blank tube

† The supernatant from this tube, after recentrifugation, was treated with 0 5 ml of the rabbit anti Pn III serum dilution, with the result given in the last column of the table

‡ One half of 0 405 (preceding column)

[5] The membranes and filtering apparatus were kindly supplied by Miss Katherine C Mills of this department

Experiment 3 —65 ml. of guinea pig serum were neutralized and filtered as in the preceding experiment The filtrate contained 200 hemolytic units per ml One half was inactivated as before Precipitin reactions were first carried out in both portions with anti Pn horse serum, which is known not to fix complement (8, 9) Anti Pn I horse serum 902 [6] containing 3 14 mg of precipitin N per ml was used after neutralization The reactions were run as follows in 50 ml. centrifuge tubes, precipitates were centrifuged after 1 hour at 20 C and washed 3 times with 10 ml of chilled saline All supernatants were recentrifuged The washed precipitates were dissolved in alkali and rinsed into 20 ml volumetric flasks Two 6 0 ml samples of each solution were analyzed

C *ml*	*30*				*1 5*
1C′ *ml*		30			
Saline *ml*	1	1	30	30	
Anti Pn I serum *ml*	1 0	1 0	1 0	1 0	
S I 0 5 mg per ml *ml*	1 0	1 0	1 0		0 05
N pptd from aliquot *mg*	{0 784 {0 790	{0 784 {0 788	{0 746 {0 746	0 002	0
N pptd from entire sample† *mg*	2 62	2 61	2 48		
N pptd per 5 ml C 1C′, saline resp *mg*	0 437	0 435	0 413		

* Qualitative test
† Less blank

The C′ and 1C′ supernatants recovered from the above reaction were then employed in a precipitin reaction with inactivated neutralized rabbit anti-egg albumin (Ea) serum 3 87 II which had been diluted with 4 volumes of saline containing 1 10 000 merthiolate [7]

No of tubes	1	1	1	1	2	3	3	3
C *ml*	5 5*	5 5*						5 5*
1C′ *ml*			5 5*	5 5			5 5*	
Anti Ea serum dilution, *ml*	1 0		1 0		1 0	1 0	1 0	1 0
Ea 0 036 mg N per ml. *ml*		0 2		0 2		1 0	1 0	1 0
Saline *ml*					6 5	5 5		
N pptd *mg*	0 016	0 016	0 018	0 010	0	{0 478 {0 478 {0 480	{0 498 {0 494 {0 502	{0 616 {0 620 {0 622
Mean	0 016		0 014			0 479	0 498	0 619
Subtraction of blank *mg*						0	0 014	0 016
Specific N pptd *mg*						0 479	0 484	0 603
Subtraction of 1C′ series value								0 484
C′1 N pptd *mg*								0 12

Hemolytic units per 5 ml. of original C	1000
† after addition of anti Pn I horse serum	730
† pptn of S I anti S I	670
† Ea anti Ea	<75

* Corresponding to 5 0 ml of original C or 1C
† Calculated to original volume

[6] Obtained through the courtesy of Dr R H Muckenfuss Miss A Walter and Dr E M Schryver of the New York City Department of Health
[7] Manufactured by Eli Lilly & Company, Indianapolis

Experiment 4.—Experiment 3 was repeated with omission of the filtration and use of only one half the quantities of antipneumococcus horse serum and S I. The amounts of nitrogen precipitated in the presence of saline, 32 ml. of iC′, and 32 ml. of C′ were 1.136, 1.276, and 1.344 mg., respectively, or 0.210, 0.236, and 0.247 mg. per aliquot portion (6.5 ml. out of a total of 35 ml.) used in the second half of the experiment with Ea-anti-Ea. In this portion of the experiment the mean values for N precipitated in the presence of saline, iC′, and C′ were 0.482, 0.500, and 0.644 mg., respectively, giving 0.144 mg. as the amount of C′1 N found. In Table I there is added to this amount the 0.01 mg. C′1 N apparently brought down by the corresponding aliquot of the S I anti-Pn I horse specific precipitate. 6.5 ml. of the supernatant from the S anti S precipitate contained only 1100 hemolytic "units" although the number in the complement originally taken was 1480.[8] This last number is used in Table I in calculating the complement N precipitated per 1000 "units." After precipitation of the Ea anti-Ea the supernatant contained ≪40 "units."

TABLE I

Complement Nitrogen Added to Specific Precipitates

Experiment No.	Total nitrogen precipitated		Total complement N precipitated (C 1 N)	Complement N precipitated (C 1 N)		
	in presence of active complement	in presence of inactivated complement		per 1000 hemolytic units taken up	per 5 ml. active guinea pig serum	per 1 ml. active guinea pig serum
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.720	0.600	0.120	0.096	0.120	0.024
2	0.565	0.405	0.160	0.132	0.160	0.032
3	0.603	0.484	0.119	0.119	0.119	0.024
4	0.644	0.500	0.154*	0.104	0.131	0.026
5	0.773	0.611	0.162	0.141	0.162	0.032

* 0.144 + 0.01 apparently added to the mutual S I anti Pn I horse serum specific precipitate.

Experiment 5.—84 ml. of neutralized guinea pig serum were used, of which 28 ml. were left in the native state (C′), 28 ml. were inactivated in the usual way (iC′), and 28 ml. were warmed to 56°C. with the thermometer in the serum and left at 56° for 5 minutes, the serum being exposed to 50° and over for a total of 10 minutes (i′C′). Both iC′ and i′C′ were incapable of hemolyzing sensitized sheep cells. In order to test the validity of averaging the blanks, as had been done previously, a third blank tube was added in each series containing only the appropriate portion of guinea pig serum and saline. Owing to the increased number of blanks the precipitin determinations were run only in duplicate. After the original set of tubes and the supernatants had been centrifuged the supernatants from each set of antigen and antibody blanks were combined, as indicated in the protocol, giving another set of independent determinations with twice the quantity of C′, iC′, and i′C′. After 1 to 1½ hours these were also centrifuged and the analyses and hemolytic tests completed. The rabbit serum and dilution used were the same as in Experiment 1. The S III solution contained 0.042 mg. of S III per ml.

[8] When titrated as indicated in Table I of the following paper the original complement and the supernatant from the S I precipitate showed little difference.

No of tubes	2	1	1	1	1	1	1	1	1	1	2	2	2	2	1	1	1
C *ml*		5 0	5 0	5 0										5 0	10		
1C *ml*					5 0	5 0	5 0						5 0			10	
1 C *ml*								5 0	5 0	5 0		5 0					10
Serum dilu tion *ml*	1 0	1 0			1 0			1 0			1 0	1 0	1 0	1 0	½	1	1
S III *ml*			1 0			1 0			1 0		1 0	1 0	1 0	1 0	1	1	1
Saline *ml*	1			1 0			1			1	5						
N pptd *mg*	0	0 014	0 014	0 014	0 018†	0 022	0 024	0 070	0 020	0 026	∫0 602 ∫0 596	∫0 686 ∫0 692	∫0 626 ∫0 642	∫0 790 ∫0 784	0 846	0 624	0 740
Mean *mg*		0 014			0 023			0 022			0 599	0 689	0 634	0 787			
S btraction of blank *mg*											0	0 012	0 0-3	0 014			
Specific N pptd *mg*											0 599	0 667	0 611	0 773	0 8631		
Subtraction of C N *mg*												0 611		0 611	0 611		0 611
C 1 N pptd *mg*												0 06		0 16	0 25		0 13

Hemolytic units taken in main series	1180
left in C′ supernatants	30
taken in combined blank supernatants	2360
left	85

* The supernatants from each pair of S III and serum blanks were recentrifuged and then combined and mixed in a third set of tubes Unavoidable losses would probably increase N pptd by 3 to 5 per cent

† Omitted as Kjeldahl flask bumped during digestion

‡ Corrected for 0 14 ml removed for hemolytic test from each blank supernatant before mixing Deducting the value 0 624 in the next column from this gives 0 24 in excellent agreement and is perhaps more accurate since double quantities were used

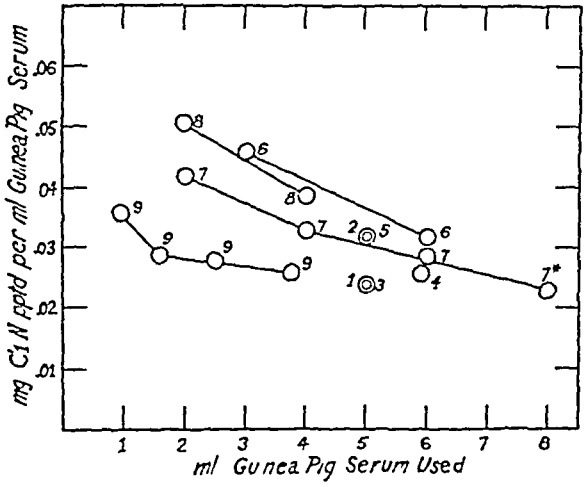
Experiment 6 —The guinea pig serum used was not neutralized Titer 400 units per ml Possibly owing to the high activity, inactivation for 35 minutes at 56 destroyed only one half of the combining power although hemolytic activity had entirely disappeared For this reason the 1C N value was calculated by addition of 0 01 mg of N for each 3 ml of guinea pig serum used to the amount of N precipitated by S III from anti S III diluted with saline, this being approximately the increase between the salt and 1C′ runs calculated from Experiments 1 to 5 With this as a basis it was possible to estimate C′1 N from duplicate precipitations of S III anti S III in the presence of 3 0 and 6 0 ml of the active guinea pig serum It will be noted from Text fig 1 that more C′1 N per ml. was precipitated from 3 ml. of guinea pig serum than from 6 ml (0 046 and 0 032 mg respectively) although judging by the hemolytic activity less than 40 units of complement remained in the latter supernatant

3 0 ml. portions of the same pool were also run at 0 for 24 hours but only 0 037 mg of C 1 N per ml were added Possibly maximal values would have been reached in 48 hours as in the precipitin reaction (5)

Experiment 7 —Portions of another guinea pig serum pool were tested untreated (active pH 8 07 inactivated pH 8 33) and neutralized with 0 15 N HCl in 0 9 per cent saline (active pH 6 75 inactivated, pH 6 78 after addition of a little more acid to replace the CO_2 evolved) Titer during experiment untreated 290 units,' neutralized 250

"units" In this instance, also, inactivation for 30 minutes after the serum temperature reached 56° was insufficient to destroy all combining component, although inactivation in this respect was nearly complete in the alkaline, untreated portion 1C′ N was calculated as in the preceding experiment With 4 0 ml of untreated active guinea pig serum 0 035 mg of C′1 N per ml were precipitated, while from 6 0, 4 0, and 2 0 ml of the neutralized pool, 0 029, 0 033, and 0 042 mg of C′ N per ml were precipitated by S III-anti-S III, corrected for the dilution attendant upon neutralization Thus the same decrease per milliliter with increasing amounts of complement was noted in this instance (see text-figure) although in all cases practically all hemolytic activity was removed by the specific precipitate

With this pool, also, a 24 hour run at 0° yielded slightly lower results than at room temperature



TEXT-FIG 1 Amount of complement combining component nitrogen (C′1 N) precipitated as a function of the volume of guinea pig serum used

The numbers at each point refer to the experiment 7* = combined serum and S III blank supernatants

Experiment 8—Complement not neutralized, merthiolate,[7] 1 10,000, added before allowing to stand in the cold overnight Titer 200 "units" Heated portion fully inactivated with respect to combining component in 50 min at 56° Data for 2 0 and 4 0 ml of complement are plotted in the text-figure Values in this series were compared undiluted and at a volume of 10 ml, 0 9 per cent saline being the diluent The additional electrolyte slightly increased the amount of C′1 N found throughout the series, but the differences were scarcely outside the analytical error

3 0 ml of the supernatants from the precipitation of *S III* anti-*S III* in 4 0 ml of undiluted active[9] and inactivated complement were again set up, in duplicate, with

[9] The supernatant from the specific precipitation in active complement was, of course, inactive hemolytically

appropriate blanks with 1 0 ml of anti S III and S III dilutions The amounts of nitrogen precipitated were saline anti S III, S III 0 538 mg , 1C′, anti S III, S III, 0 552 mg , C , anti S III, S III 0 546 mg Only traces of non specific nitrogen were removed from the C′ and 1C′ supernatants, showing the C′1 N to have been completely absorbed by the first precipitation

Experiment 9 —Complement neutralized as in Experiment 7 merthiolate added Titer 170 'units per ml Inactivation as in Experiment 8 Data plotted in text figure

Fractionated Complement —Complement was separated by dialysis or by dilution with water and precipitation with CO_2 into the precipitable combining component and so called "end piece " The former, redissolved in saline, added nitrogen to antigen antibody precipitates, much as did whole guinea pig serum, while the supernatant or "end piece" behaved like inactivated complement This phase of the work, which should be of value in the isolation of complement components, will be dealt with in a separate communication

DISCUSSION

It was apparent even in the very first of the experiments described above that active complement affected specific precipitation differently from in activated complement or a corresponding amount of saline Particulation was greatly delayed in the tubes containing active complement and the precipitate remained more finely divided and settled less readily than in the other tubes In later experiments, in which relatively larger amounts of saline were used as diluent, this effect was less marked

The protocols of the runs, the summary in Table I, and the text figure all show that appreciable quantities of nitrogen may be added to specific precipitates from rabbit antisera when these are formed in the presence of 2 ml or more of active complement or alexin Deposition of the same quantity of antigen antibody nitrogen in the presence of complement fully inactivated by heat results in the separation of little or no more nitrogen than is precipitated when a similar volume of physiological salt solution is used as diluent Proportions of reactants were so chosen that substantially all of the hemolytic activity of the complement disappeared during the precipitation If, therefore, all of the nitrogen added by the active complement, as in Experiment 1, were actually derived from this substance, the increase would serve as a measure, in weight units, of complement as defined by Muir (4) or of one or more of its components, chiefly the so called "mid piece," if complement is defined in terms of its hemolytic activity (10)

It might be objected, however, that in Experiment 1 the increased nitro-

gen or a part of it was due, not to the active complement that disappeared from solution during the precipitation, but to an invisible, non-centrifugable nitrogenous component of guinea pig serum which bore no relation to complement and which dissolved irreversibly when the serum was inactivated by heating at 56°C Experiment 2 was carried out in order to test this possibility It will be noted that a relatively large amount of guinea pig serum was filtered through gradocol membranes of 700 mμ average pore diameter with little loss of complement activity The filtration should, of course, have removed any suspended solid such as the hypothetical substance in question In spite of this, even more nitrogen was added to a smaller quantity of specific precipitate by the active complement pool used in this instance

Since excess antibody was employed in the precipitin estimations, it might also be objected that active complement might permit antigen to combine more completely with antibody and so cause an increase in antibody nitrogen to be mistaken for C'1 N Several types of experiment showing this objection, also, to be invalid, will be reported in another connection

Experiment 3 was designed to test another possible objection, namely, that the added nitrogen might not be due to complement itself, but to an easily adsorbable soluble substance capable of adding to any precipitate formed in the solution It was recalled that specific precipitates from antipneumococcus horse sera do not fix complement (8, 9) However, their general similarity to the rabbit precipitates made it appear possible that any easily adsorbable substance would be removed independently of the complement Then, if the complement remained, and were specifically bound by an antigen-rabbit antibody precipitate formed in the supernatant any added nitrogen would seem more rigorously due to the complement itself In order to repeat the test for the other interfering substance postulated in Experiment 2 the guinea pig serum pool was first filtered through gradocol membranes of 700 mμ A P D before addition of Type I antipneumococcus horse serum containing antibody equivalent to the amount of rabbit antibody to be used later It will be noted from the protocol (page 685) that practically identical quantities of nitrogen were precipitated by S I from the active and inactivated complement-anti-Pn I horse serum mixtures, a behavior in sharp contrast to that of the S III-anti-S III rabbit system That suitably prepared S I absorbs complement with rabbit anti-S I had already been shown (9) and this was confirmed with the preparation used in the present experiments It will be noted, also, that the nitrogen precipitated from the active and inactivated complement tubes exceeded that

from the saline tube by about 0 02 mg per 5 ml of active or inactivated complement, an amount comparable to the difference between the saline and inactive complement controls in the other experiments This small difference may partly represent lipid nitrogen (*cf* Horsfall and Goodner (11)) carried down by the specific precipitates from the guinea pig serum, the more so as these precipitates, dissolved in alkali, often formed initially slightly turbid solutions The difference is also due in part to traces of non specific nitrogen remaining in spite of the three washings (*cf* last part of Experiment 8)

The egg albumin rabbit anti Ea precipitates formed in the second half of the experiment (page 685) not only dissolved in alkali to form clear solutions, indicating that lipid had been removed by the first precipitate, but also showed a difference between the active and inactivated complement tubes entirely comparable with that in Experiments 1 and 2 It would seem difficult to explain this outcome on any basis other than that the nitrogen difference was due to the active complement taken up The experiment was repeated (No 4) and gave a similar result

It was noted in the experiments with antipneumococcus horse serum that the number of hemolytic "units" was reduced by about one quarter by mere addition of the horse serum and by about one third after the precipitation with S I even though the precipitate failed to add a measurable quantity of complement nitrogen Since the amount of complement nitrogen expected from the original number of "units" was added to the rabbit specific precipitate subsequently formed in the active supernatant it would seem that the anticomplementary action of the horse serum was directed against a property concerned with hemolysis rather than against the combining property of the complement If this were true the present method for the estimation of complement or its combining component would not only supply measurements in weight units but would also provide an independent means of measurement by which factors controlling the hemolytic properties of complement could be more accurately defined

While it is hoped to carry out a detailed study along these lines in the near future, Experiment 5 was undertaken as a test of the feasibility of such a study It was also desired to check the practice, followed up to this point, of averaging the blank values obtained on addition of antigen or antibody separately to the complement samples It is evident from the protocol that this procedure is justifiable, since the blanks obtained in this way did not differ from those to which saline alone had been added [10]

[10] This has also been confirmed in a subsequent run

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QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

II The Interrelation of Complement with Antigen Antibody Compounds and with Sensitized Red Cells*

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In the preceding paper (1) a method was described by which complement, or that portion of it which combines with antigen antibody precipitates, could be measured in weight units, that is, in absolute rather than relative terms It was estimated that the samples of pooled guinea pig sera analyzed contained from 0 04 to 0 06 mg of complement nitrogen per ml , correspond ing to 0 25 to 0 4 mg of complement combining component, if the substance is a globulin, as seems certain (2–4) With the aid of these figures it is now possible to define quantitative relations, under the conditions used, between complement, antigen, antibody including hemolysin, and the sensitized sheep red cell

It has long been known that titration of varying amounts of hemolysin against varying quantities of complement yields dilution end point curves roughly of parabolic form This renders difficult the establishment of definite "units" and the determination of the minimal amounts of antigen, antibody, hemolysin, and complement effective in complement fixation tests Full data are therefore given below, and from these it should be possible to derive any conventional "unit" desired whether it be based on initial, 50 per cent, complete hemolysis, or some other factor While such "units" have hitherto been of great practical value they are not essential to a con sideration of the relations between the reacting components in complement fixation and are therefore not used in the present discussion

EXPERIMENTAL

The Relation between Complement, Hemolysin, and Red Cells —The rabbit anti sheep cell hemolysin used in Experiments 1, 2, and 3 of the preceding

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital

TABLE II

Titration of Complement and Hemolysin

Hemolysin	Hemolysin N	Complement ml				
		10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C 1 N = 0 13γ	$1\ 67 \times 10^{-3}$ C 1 N = 0 08γ
ml $\times 10^{-5}$	γ					
6	0 03	c	c	c	c	ac
4	0 02	c	c	c	c	m
3	0 015	c	c	c	ac	m
2	0 01	c	c	ac	m	sl
1 5	0 008	c	c	st	sl	tr
1	0 005	st	m	sl	0	0
0 75		m	sl	sl	0	0
0 5		sl	tr	0	0	0
0		0	0			

Symbols 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis

TABLE III

Fixation of Complement by S III[3] and Antipneumococcus Type III Rabbit Serum

Antibody N	10×10^{-5} ml hemolysin or 0 05γ hemolysin N								
	0 1γ S III				0 01γ S III				
	Complement ml				Complement ml				
	10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C 1 N = 0 13γ	10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C 1 N = 0 13γ
γ								
5	0 0	0 0	0 0	0 0	st c	tr sl	0 0	0 0
3	0 0	0 0	0 0	0 0	m c	0 0	0 0	0 0
2	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
1	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
0 6	0 0	0 0	0 0	0 0	0 c	0 0	0 0	0 0
0 4	0 st	0 0	0 0	0 0	0 ac	0 0	0 0	0 0
0 2	tr c	0 0	0 0	0 0	0 st	0 0	0 0	0 0
0 12	ac c	0 m	0 0	0 0	ac c	0 m	0 0	0 0
0 08	c c	st c	tr m	0 0	c c	m c	0 m	0 0
0 04	c c	c c	m c	0 m	c c	c c	m ac	0 0
0 024	c c	c c	ac c	0 c	c c	c c	m c	0 tr
0 016	c c	c c	c c	0 c	c c	c c	ac c	sl
0	c c	c c	c c	0 st	c c	c c	c c	0 sl
	3×10^{-5} hemolysin or 0 015γ hemolysin N							
5	0 0	0 0	0 0		c c	tr sl	0 0	
3	0 0	0 0	0 0		st ac	0 tr	0 0	
2	0 0	0 0	0 0		m st	0 0	0 0	
1	0 0	0 0	0 0		m st	0 0	0 0	
0 6	0 0	0 0	0 0		sl m	0 0	0 0	
0 4	0 0	0 0	0 0		0 sl	0 0	0 0	
0 2	m st	0 0	0 0		tr m	0 0	0 0	
0 12	ac c	m ac	tr sl		st c	sl m	0 0	
0 08	c c	c c	sl st		c c	st ac	sl m	
0 04	c c	c c	c c		c c	c c	c c	
0	c c	c c	c c		c c	c c	c c	

Symbols 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis

45 minutes at 37 C each tube received a mixture of 0 1 ml of 4 per cent sheep red cell suspension and 0 1 ml containing the volume of hemolysin given in the subheading of Table III Control rows of tubes containing saline instead of S III were included with each complement dilution These showed complete hemolysis at the end except for the $2\ 5 \times 10^{-3}$ complement dilution which was unhemolyzed at the first reading and not entirely hemolyzed at the end This dilution is therefore not used in computing reacting quantities and is included in the table both to show the limit of sensitivity of this mode of titration and to indicate an uncertainty in the calculations If 0 13 γ of C'1 N did not always suffice for complete hemolysis under the conditions described it is equally uncertain whether all of the C 1 N present in the system was taken up in complete inhibition of hemolysis At this stage it would appear premature to attempt any correction for these unknown and apparently variable factors especially as this would involve deductions at each end of the hemolysis scale which might be equal and thus cancel out Any lack of homogeneity in the complement combining component would also have to be considered in this connection

In each column of Table III the first row of symbols indicates the reading after all control tubes except those mentioned above were completely hemolyzed. The second row of symbols shows the reading after 10 minutes additional standing at room temperature after which there was no appreciable change The first readings appear to give more sensitive indications of zone effects than the final ones and are included for possible future reference

It is evident from Table III, subject to the uncertainties previously mentioned, that as little as 0 12 γ of anticarbohydrate N in antipneumococcus Type III rabbit serum may take up as much as 0 2 γ of complement N, or complement combining component (C'1) N in the reaction of 0 1 γ of S III with the antibody Similarly 0 2 γ of anticarbohydrate N and 0 1 γ of S III take up 0 3 γ of C'1 N in combining At these high dilutions the solutions remain crystal clear until the hemolytic system is added With the above proportions of S III and antibody the sensitivity of the test for complete fixation of complement decreases to 0 2 γ of antibody N if the hemolysin in the hemolytic system is reduced from 0 05 γ to 0 015 γ of N This difference in sensitivity vanishes when only 0 01 γ of S III is used as antigen possibly because the S III antibody proportions are then more nearly equivalent at this end of the reaction range (10) The limit of sensitivity as regards antibody N remains the same, however, as at the higher level of S III concentration It is also to be noted that 0 01 γ of S III does not suffice for the complete fixation of as much as 0 5 γ of C'1 N between the limits of antibody concentration used, while a definite zone of complete fixation occurs with 0 3 γ of C'1 N extending over a 10 to 15 fold range of antibody concentration In the series in which 0 1 γ of S III was used, however, 0 5 γ of C'1 N was readily taken up at antibody N levels of 0 4 γ and above

In Table IV are given similar data for Ea and anti Ea rabbit serum This

TABLE II

Titration of Complement and Hemolysin

Hemolysin	Hemolysin N	Complement ml				
		10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C 1 N = 0 13γ	$1\ 67 \times 10^{-3}$ C 1 N = 0 03γ
ml $\times 10^{-5}$	γ					
6	0 03	c	c	c	c	ac
4	0 02	c	c	c	c	m
3	0 015	c	c	c	ac	m
2	0 01	c	c	ac	m	sl
1 5	0 008	c	c	st	sl	tr
1	0 005	st	m	sl	0	0
0 75		m	sl	sl	0	0
0 5		sl	tr	0	0	0
0		0	0			

Symbols 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis

TABLE III

Fixation of Complement by S III[3] and Antipneumococcus Type III Rabbit Serum

Antibody N	10×10^{-5} ml hemolysin, or 0 05γ hemolysin N							
	0 1γ S III				0 01γ S III			
	Complement, ml				Complement ml			
	10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C'1 N = 0 13γ	10×10^{-3} C'1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C 1 N = 0 13γ
γ								
5	0 0	0 0	0 0	0 0	st c	tr sl	0 0	0 0
3	0 0	0 0	0 0	0 0	m c	0 0	0 0	0 0
2	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
1	0 0	0 0	0 0	0 0	sl c	0 0	0 0	0 0
0 6	0 0	0 0	0 0	0 0	0 c	0 0	0 0	0 0
0 4	0 st	0 0	0 0	0 0	0 ac	0 0	0 0	0 0
0 2	tr c	0 0	0 0	0 0	0 st	0 0	0 0	0 0
0 12	ac c	0 m	0 0	0 0	ac c	0 m	0 0	0 0
0 08	c c	st c	tr m	0 0	c c	m c	0 m	0 0
0 04	c c	c c	m c	0 m	c c	c c	m ac	0 0
0 024	c c	c c	ac c	0 c	c c	c c	m c	0 tr
0 016	c c	c c	c c	0 c	c c	c c	ac c	sl
0	c c	c c	c c	0 st	c c	c c	c c	0 sl
3×10^{-5} hemolysin or 0 015γ hemolysin N								
5	0 0	0 0	0 0		c c	tr sl	0 0	
3	0 0	0 0	0 0		st ac	0 tr	0 0	
2	0 0	0 0	0 0		m st	0 0	0 0	
1	0 0	0 0	0 0		m st	0 0	0 0	
0 6	0 0	0 0	0 0		sl m	0 0	0 0	
0 4	0 0	0 0	0 0		0 sl	0 0	0 0	
0 2	m st	0 0	0 0		tr m	0 0	0 0	
0 12	ac c	m ac	tr sl		st c	sl m	0 0	
0 08	c c	c c	sl st		c c	st ac	sl m	
0 04	c c	c c	c c		c c	c c	c c	
0	c c	c c	c c		c c	c c	c c	

Symbols 0 = no hemolysis, tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis

45 minutes at 37 C each tube received a mixture of 0 1 ml of 4 per cent sheep red cell suspension and 0 1 ml containing the volume of hemolysin given in the subheading of Table III Control rows of tubes containing saline instead of S III were included with each complement dilution These showed complete hemolysis at the end except for the $2\,5 \times 10^{-3}$ complement dilution, which was unhemolyzed at the first reading and not entirely hemolyzed at the end This dilution is therefore not used in computing reacting quantities and is included in the table both to show the limit of sensitivity of this mode of titration and to indicate an uncertainty in the calculations If 0 13 γ of C 1 N did *not always suffice for complete hemolysis under the conditions described it is equally* uncertain whether all of the C 1 N present in the system was taken up in complete inhibition of hemolysis At this stage it would appear premature to attempt any correction for these unknown and apparently variable factors especially as this would involve deductions at each end of the hemolysis scale which might be equal and thus cancel out Any lack of homogeneity in the complement combining component would also have to be considered in this connection

In each column of Table III the first row of symbols indicates the reading after all control tubes except those mentioned above were completely hemolyzed The second row of symbols shows the reading after 10 minutes additional standing at room temperature, after which there was no appreciable change The first readings appear to give more sensitive indications of zone effects than the final ones and are included for possible future reference

It is evident from Table III, subject to the uncertainties previously mentioned, that as little as 0 12 γ of anticarbohydrate N in antipneumococcus Type III rabbit serum may take up as much as 0 2 γ of complement N, or complement combining component (C'1) N in the reaction of 0 1 γ of S III with the antibody Similarly 0 2 γ of anticarbohydrate N and 0 1 γ of S III take up 0 3 γ of C'1 N in combining At these high dilutions the solutions remain crystal clear until the hemolytic system is added With the above proportions of S III and antibody the sensitivity of the test for complete fixation of complement decreases to 0 2 γ of antibody N if the hemolysin in the hemolytic system is reduced from 0 05 γ to 0 015 γ of N This difference in sensitivity vanishes when only 0 01 γ of S III is used as antigen possibly because the S III antibody proportions are then more nearly equivalent at this end of the reaction range (10) The limit of sensitivity as regards antibody N remains the same, however, as at the higher level of S III concentration It is also to be noted that 0 01 γ of S III does not suffice for the complete fixation of as much as 0 5 γ of C'1 N between the limits of antibody concentration used, while a definite zone of complete fixation occurs with 0 3 γ of C'1 N extending over a 10 to 15 fold range of antibody concentration In the series in which 0 1 γ of S III was used however, 0 5 γ of C'1 N was readily taken up at antibody N levels of 0 4 γ and above

In Table IV are given similar data for Ea and anti Ea rabbit serum This

portion of the experiment was carried out in the same way as the preceding part First readings, as recorded in Table III, are omitted since there was little difference except in column 2 of the table A series of titrations was also carried out with 0 01 γ of Ea N, but only traces of complement were taken up This is in accord with the far lower antibody N Ea ratios which obtain in the Ea-anti-Ea precipitin reaction (11) than the ratios characteristic of the S III-anti-S III reaction (10) Nevertheless, in the Ea-anti-Ea reaction, a typical antigen-antibody system, the smallest amount of antibody N detectable with certainty was 0 12 γ, as in the S-anti-S

TABLE IV

Fixation of Complement by Egg Albumin and Anti-Egg Albumin Rabbit Serum

Antibody N	0 1γ egg albumin N						
	10×10^{-6} ml hemolysin, or 0 05γ hemolysin N				3×10^{-6} ml hemolysin or 0 015γ hemolysin N		
	Complement ml				Complement, ml		
	10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C 1 N = 0 2γ	$2\ 5 \times 10^{-3}$ C'1 N = 0 13γ	10×10^{-3} C 1 N = 0 5γ	$6\ 25 \times 10^{-3}$ C 1 N = 0 3γ	4×10^{-3} C'1 N = 0 2γ
γ							
5	m	0	0	0	0	0	0
3	m	0	0	0	0	0	0
2	0	0	0	0	0	0	0
1	m	0	0	0	0	0	0
0 6	m	0	0	0	0	0	0
0 4	m	0	0	0	0	0	0
0 2	st	0	0	0	0	0	0
0 12	c	sl	0	0	m	0	0
0 08	c	c	sl	0	st	m	m
0 04	c	c	c	sl	c	ac	st
0 024	c	c	c	ac	c	c	ac
0	c	c	c	st	c	c	ac

Symbols as in Tables II and III

system, and again the antigen-antibody complex was found capable of taking up at least an equal weight of complement N

Additional Data on Complement Fixation —In a number of earlier experiments the quantity of complement was held constant at 0 25 ml of a 1 10 dilution, or approximately 1 3 γ of C'1 N, while 3 to 10 minimal hemolytic doses of hemolysin were used Under these conditions the maximum sensitivity for anti-S I was reached at 1 8 γ of antibody N with 0 25 γ of S I, but if as little as 0 025 or 0 016 γ of S I was used the minimum amount of antibody N which completely fixed 1 3 γ of C'1 N was 3 γ Later experiments with 0 5 γ of C'1 N and 0 1 γ of S I showed a lower limit of 0 2, 0 4, and 0 4 γ of antibody N with three different antisera, while with 0 01 γ

of S I the values were 0 12 or less, 0 8, and 0 8 γ With S II and an anti pneumococcus Type II rabbit serum the lowest limits of complete fixation of approximately 0 5 γ of C'1 N were 1 5, 0 5, and 0 3 γ of antibody N with 1, 0 1, and 0 01 γ of S II respectively Repetition of the experiment with another serum gave 1 1, 0 3, and 0 3 γ of antibody N

A sample of purified Type I pneumococcus rabbit anticarbohydrate, 193B, was prepared by dissociation with barium hydroxide (12) after previous extraction of a portion of the washed precipitate with 15 per cent salt solution The solution contained 0 44 mg of N per ml , of which 0 4 mg was antibody N A dilution containing 10 γ of antibody N fixed 1 3 γ of C'1 N completely with 2 5 γ of S I as antigen The lower limit of sensitivity for antibody was not reached, as smaller amounts of S I were not tested With the same antibody solution, 20 γ of antibody N fixed 1 3 γ of complement N completely with S I quantities ranging from 20 γ down to 0 03 γ Similar proportions and limits were found in the S III anti S III system, in which 15 γ of antibody N (diluted antiserum) fixed 1 3 γ of C'1 N with quantities of S III ranging from 25 γ down to 0 025 γ As nearly as can be deduced from their studies with larger "units," Goodner and Horsfall (13) found combining proportions of similar range and order of magnitude with S I and antipneumococcus Type I rabbit serum of known antibody content

With the corresponding homologous type specific polysaccharides two antipneumococcus Type III rabbit sera and one of two Type VIII sera gave fixation of 1 3 γ of C'1 N in and below the zone of visible precipitation, but cross reactions between S III and anti S VIII or S VIII and anti S III could not be demonstrated either by precipitation or by complement fixation On the other hand S II precipitated and fixed complement with rabbit antiserum to Friedlander's bacillus Type B over a narrower range than the Friedlander B polysaccharide, but the latter substance failed to precipitate or fix complement with the rabbit anti Pn II serum used although this contained 1 74 mg of anti S II per ml (*cf*, also 14)

Highly purified bovine antipneumococcus Types I and II antibody solutions (12) also showed relations between antigen, antibody, and complement similar to those exhibited by antibody formed in the rabbit With 0 04 γ of S I, bovine anti S I fixed 1 3 γ of C'1 N down to 3 6 γ of antibody N, while bovine anti S II showed a similar lower limit with 0 02 γ of S II This result indicates that the high molecular weight of horse antipneumococcus antibodies (5, 6) is not responsible for the failure of these antibodies to fix complement, since the bovine antipneumococcus antibody has the same high molecular weight (6)

DISCUSSION

It was shown in the preceding paper that the combining component of guinea pig complement could be measured in weight units and that more than 40 per cent of complement, by weight, could be added to antigen-antibody precipitates with maintenance of volumes of the reacting components at levels convenient for precise analytical measurement However, it was scarcely feasible to use larger and larger volumes of guinea pig serum in order to determine the maximum amount of complement capable of reaction with known quantities of antigen and antibody Instead, the usual technique of the complement fixation test, with its great delicacy and reproducibility under strictly controlled conditions, appeared capable of affording the necessary information, and could, by virtue of this very delicacy, be used to determine at the same time the lower limits of reactivity and the combining proportions of the reacting components By the use of antigen and antibody solutions of known content and with the aid of the values for complement obtained in the preceding paper the actual quantities of antigen, antibody, and complement could be calculated in weight units for each significant dilution and mixture In several experiments the number of red cells in the hemolytic system was counted and the amount of hemolysin estimated [1]

Since the hemolysis of sensitized red cells by complement depends upon other factors in guinea pig serum as well as the combining component of complement (2, 3, 4, 15) it is impossible to define the mechanism of hemolysis solely on the basis of the number of red cells and the actual quantities of hemolysin and complement combining component The cell count is easily made, however, and the other two variables may now be estimated with a fair degree of certainty Their interrelationships, under the conditions used, are set forth in Table I Additional data on the relative amounts of hemolysin and complement combining component required as the test of complete hemolysis approaches its limit of delicacy, will be found in the first portion of Experiment 10 It is evident that at extreme dilutions the actual amounts of hemolysin and complement combining component approach equality, but that as the test is ordinarily carried out far more combining component than hemolysin is present The latter may be considered as actually combined with the red cells in the experiments summarized in Table I as only twice the minimum sensitizing quantity was used, but it seems probable that, except at the highest dilutions of guinea pig serum shown in Table II, complement was present in excess Possibly only an amount equal to the hemolysin, or a small multiple of this amount, entered into actual combination with the sensitized cells (*cf* 16) For this

reason, if any portion of the complement functions enzymatically in hemolysis, it is probably not the combining component, for at least as much of this protein as hemolysin must be present This does not resemble catalysis Moreover, the combining component of complement actually unites in stable union with sensitized red cells, as with other antigen antibody systems (17) [4]

The available data regarding the antibody molecule (6, 7) are such that an uncertainty remains regarding its shape The experimental value for the frictional coefficient or asymmetry factor, however, permits the calculation of molecular areas on the assumption of either oblate or prolate spheroidal shape The area occupied by such molecules on the red cell surface would depend upon their attachment in either the endwise or lateral position In Table 1 *b* calculations of the occupied surface are therefore given for each position of the oblate or prolate spheroidal molecule Intermediate positions are also conceivable Depending, then, on which calculation is used, the experimental data indicate that roughly from 0 8 to 28 per cent of the sheep cell surface is occupied by hemolysin under the conditions employed Since two "units" of hemolysin were actually used, complete hemolysis is possible with 0 4 to 14 per cent of the cell surface combined with antibody While these figures are not to be taken too literally they do at least indicate by roughly a power of ten that sensitization need not involve a coating of the entire red cell surface The findings are therefore in accord with the "key spot" theory of Abramson (18)

Brunius (19) has calculated the number of the Forssman antibody molecules required to sensitize a single red cell for hemolysis, finding the number as only 30 and the fraction of red cell surface covered as 0 001 per cent The area calculations were made on a different basis, but the number of molecules of hemolysin was calculated from a single preparation which showed about ten times the activity per γ as of N as four other lots isolated by Brunius, the best preparation of Locke and Hirsch (20), and our own sera, judged by their antibody content When it is considered that the 3000 molecules of hemolysin indicated in Table I represent double the number necessary for complete hemolysis, and that Brunius used 90 per cent hemolysis as the end point, the agreement among the less active preparations is moderately close However, sheep red cell stromata may contain a number of antigens While the antibodies resulting from the injection of these antigens into rabbits are capable of combining with stromata[4] it does not follow that all of the antibodies actually prepare the cell for hemolysis The one result used by Brunius for his calculations may there

[4] C'1 N is taken up by sheep cell stromata in their reaction with hemolysin (unpublished experiment)

fore be the more significant for the process of hemolysis itself, while the larger number of molecules found in the present experiments represents combination between the red cell and total antibody under the conditions of the hemolytic test as actually carried out with rabbit hemolysin

Although the present experiments have only limited application in the study of immune hemolysis they afford a much more complete picture of the fixation of complement in certain immune reactions With the use of 50 γ as the average complement combining component nitrogen content of guinea pig serum (1) it is possible to interpret complement fixation tests carried out on a number of sera of known antibody content (for example, Experiment 10) From the limiting dilutions of complement, antigen, and antibody at which the quantity of complement used in each instance was completely taken up it would seem evident that at least as much complement nitrogen as antibody nitrogen may enter into the reaction Experiments over a wide range with S I and anti-S I also showed that quantities of complement larger than at the limiting dilution could be fully taken up only in the presence of larger amounts of antibody, and that the antigen could be varied relatively independently above a minimum value It is also clear that the final colloidal state of the system failed to influence the proportions in which complement combined, since the quantities of complement N added to antigen-antibody mixtures yielding specific precipitates with 0 4 to 0 6 mg of N were of the same relative magnitude as in the clear solutions containing only fractions of a γ of reactants A chemical, rather than physical, explanation for the fixation of complement is therefore indicated and Ehrlich's concept of complement activity is confirmed in this respect both qualitatively and quantitatively

At the time Ehrlich's theories were proposed the nature of antigens and antibodies was uncertain and methods of measurement were purely relative After the introduction of quantitative, absolute methods (21) a large body of precise information regarding immune reactions was assembled, and with the recognition of the protein nature of antibodies it became possible to formulate chemical theories of antigen-antibody reactions which were in accord with modern concepts of the structures of the reacting substances (21, 22) Now that the combining component of complement has been added to the list of immune substances measurable in weight units it is possible to put these theories to the severe test of their adaptability to the inclusion of complement, hitherto neglected for the sake of simplicity

It was shown by the work of this laboratory that the precipitin (23) and agglutinin (24) reactions might be quantitatively expressed by equations derived from the mass law Chief among the assumptions made was that

both antigen and antibody were multivalent with respect to each other, that is, that each possessed two or more groupings reactive with the other After the molecular weights of antibodies became known (5, 6) it was possible to assign empirical formulas to specific precipitates formed at certain reference points in the precipitin reaction range (25) Now that both the molecular weight[5] and the reacting quantities of the combining component of complement are known it is possible to fit this component into the above quantitative theory with little difficulty

In a number of papers (23, 11, 21 *b*, and others) two dimensional representations of three-dimensional aggregates formed by the union of multivalent antigen with multivalent antibody were depicted somewhat as follows

[1]

in which S represents specific polysaccharide or antigen and A represents antibody, or the compounds Ea_nA_{4+2} and (EaA_2) in the antibody excess and equivalence zones respectively (21 *b*)

[2]

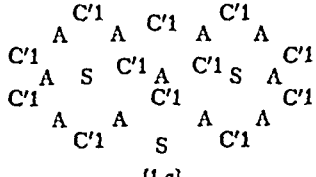
[3]

In the last two formulas the valence of Ea is taken as 6 and that of A limited to 2 for simplicity A somewhat similar scheme has been suggested by Marrack (22) and more recently an analogous one by Pauling (26), in which the valence of antibody was limited to two

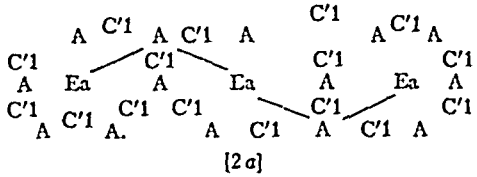
The assumption is now made that the combining component of complement, whether it possesses a single reactive grouping, or whether like antigen and antibody it carries two or more reactive groupings, is capable only of loose, easily dissociable combination with dissolved antibody This is in accord with numerous observations that complement is not fixed by antigen or antibody alone (2, 3) How then, is it possible to account for the firm fixation of amounts of complement equimolecular[5] with those of antibody by antigen antibody combinations whether or not these actually separate from solution?

[5] Private communication from Professor E E Ecker and Dr L Pillemer (*cf* Table I *a*)

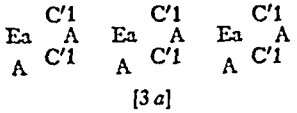
It will be recalled that in the development of the quantitative theory of the precipitin reaction (21, 23) it was assumed that multivalent antigen combined with multivalent antibody in a series of competing bimolecular reactions to build up large aggregates like those illustrated, and that the process might be well advanced before the aggregates separated from solution At high dilutions, particularly with rabbit antisera, which form specific precipitates of appreciable solubility (10, 11), the aggregates would not necessarily separate Whether or not precipitation occurs, the formation of such multimolecular aggregates would bring together and hold myriads of antibody molecules But by this act any molecules of complement combining components (C′1) present would be surrounded by antibody molecules In this way a linkage between C′1 and A, ordinarily dissociated at once when taking place between single molecules, might become stabilized when occurring between C′1 and nA Stabilization might result either through the attraction of approaching ionized groupings of opposite sign, through hydrogen bonding, through spatial accommodation of large groupings on C′1 and A, or through the presence, on C′1, as on antigen and antibody, of more than one grouping capable of reacting with A molecules brought into apposition The result might then be represented in the two-dimensional schemes, 1 *a*, 2 *a*, and 3 *a*, corresponding to 1, 2, and 3 above



[1 *a*]



[2 *a*]



[3 *a*]

An analogous instance of loose, dissociable combination, capable of conversion into firm union by addition to multivalent antigen-antibody aggregates, is found in the apparently "univalent" antibody known to

occur in many immune sera (11, 23, 27, and other papers) This residual antibody, remaining after fractional absorption of many sera with antigen, forms only easily dissociated, soluble compounds with antigen However, when multivalent, or complete, antibody is present, the "univalent" or residual antibody may add to the resulting antigen antibody aggregates in firm, relatively undissociated union and be precipitated just as is the multivalent antibody

In the above discussion and in the graphic representations complement has been considered, for simplicity's sake, solely in terms of its relation to antibody This suffices to explain its behavior provided the quantity of C'1 combined does not exceed that of A However, the data indicate that a somewhat larger proportion of C'1 may be added when relatively more complement is present It is therefore probable that, at least under such conditions, antigen molecules may participate in binding C'1 firmly in the antigen antibody aggregate, and it is not excluded that antigen plays some part under all circumstances It is known, however, (25) that throughout practically the entire range of the precipitin reaction more antibody molecules than antigen molecules are present, the proportion ranging from two- to three fold in the equivalence zone to 6 times or greater in the region of large antibody excess It would therefore seem reasonable to ascribe to the network of antibody molecules the major burden in the firm linkage of complement to antigen antibody aggregates, the more so as the complement uptake is influenced to a far greater extent by the quantity of antibody present than by the amount of antigen

Other evidence in accord with the conception of complement fixation as a consequence of aggregate formation by combination of multivalent antigen with multivalent antibody is furnished by the behavior of the pneumococcus Type III and Type VIII polysaccharide rabbit antisera reactions, and of the Type II and Friedländer Type B polysaccharide reactions in rabbit anti sera In each homologous reaction with the appropriate specific polysaccharide complement was fixed over a wide range (see page 701), extending in the Type II and Type VIII sera to high dilutions at which visible precipitation no longer occurred However, no visible cross precipitation occurred, even at relatively low dilutions, between S III and anti Type VIII sera, or between S VIII and anti Type III sera, nor was complement fixed in these cross tests On the other hand S II gave specific precipitation and complement fixation with a Friedlander B antiserum, but the Friedlander B polysaccharide neither precipitated nor fixed complement with Type II antipneumococcus rabbit serum At least in these instances the possibility of multivalent antigen antibody aggregate formation seems to

be a prerequisite for complement fixation even at high dilutions at which the aggregates fail to separate

It is, however, well known that complement is not fixed by all antigen-antibody reactions which may be expressed in terms of aggregate formation by union of multivalent antigen with multivalent antibody Although some antigen-horse antibody systems fix complement, pneumococcus specific polysaccharides react with pneumococcus anticarbohydrate from the horse and with rabbit anticarbohydrate damaged by acid (28) to form aggregates which do not bind complement The same polysaccharides react with unaltered rabbit and bovine anticarbohydrate with fixation of large amounts of C'1 Since almost all antigen-rabbit antibody systems fix C'1 it would seem possible that rabbit antibody best fulfills the steric requirements for the firm union of C'1 within the molecular network of the antigen-antibody aggregates This cannot be entirely due to the equimolecular size of C'1 and rabbit antibody, since bovine A, which also permits complement fixation, is known to have a much greater size Possibly accompanying serum lipids play a part, as postulated by Goodner and Horsfall (13)

According to these views, then, complement combining components would differ from normal globulin in the possession of one or more groupings capable of forming loose dissociable unions with individual antibody (and perhaps antigen) molecules, but yielding firm, difficulty dissociable combinations, with the exceptions noted, when surrounded by antibody (and perhaps antigen) molecules In this way the failure of C'1 to be taken up appreciably by antigen or antibody alone is readily accounted for, also the fixation of C'1 in qualitatively and quantitatively similar fashion by all but a relatively limited number of antigen-antibody combinations It is even conceivable that C'1 would unite with equal firmness with normal globulin were there a means of bringing a sufficient number of such molecules into suitable apposition and holding them there

The phenomenon of complement fixation may thus be fitted into the framework of the quantitative precipitin (and agglutinin) theory with little stretching of postulates which had already shown themselves of some utility While this affords no proof of the theory it at least justifies its use as a guide for further experiments in a field which the more conventional and alternative theories have failed to clarify

SUMMARY

1 The molecular quantities of hemolysin and complement combining component or components (C'1) involved in hemolysis have been calculated on the basis of new, quantitative, absolute methods of analysis

2 Molecular combining ratios between antigen, antibody, and C'1 have been established

3 The data are shown to be in accord with the theory of combination of multivalent antigen with multivalent antibody

4 The fixation of complement by antigen antibody combination is qualitatively and quantitatively accounted for on this basis

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INHERITANCE IN GUINEA PIGS OF THE SUSCEPTIBILITY TO SKIN SENSITIZATION WITH SIMPLE CHEMICAL COMPOUNDS

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In studies on skin hypersensitivity induced by simple substances, it has frequently been noticed by various workers that an identical treatment would lead to different degrees of sensitiveness within groups of guinea pigs even when kept under uniform conditions of diet and housing (*cf* 1, 2), similar observations have been made in experimental sensitization of human beings with simple chemical compounds, for instance by Sulzberger and coworkers (3, 4, *cf* 5, 6) The question arose whether the variations observed in the animals bespeak hereditary properties, as one might well surmise, apart from other factors, but thus far there has been no definite proof For the investigation of this matter we bred guinea pigs chosen for high (and low) susceptibility to experimental sensitization and studied susceptibility in the progeny, earlier results were reported briefly (7) The substance selected for sensitization was 2 4 dinitrochlorobenzene, which had proved to be a very suitable compound for experiments on drug allergy of the contact dermatitis type (1, 8) The experiments were devised to exclude the influence of feeding and seasonal factors (*cf* 9, 10), which are therefore outside of the scope of this paper

A particular influence of hereditary constitution is well known in certain forms of human allergy (hay fever, asthma)[1] (Cooke and Vander Veer (12), Coca and Cooke (13), Spain and Cooke (14)) Apparently this has not so far been induced experimentally in human beings in contradistinction to drug allergy in which with certain incitants sensitization is successful in almost every case (3, 15, *cf* 16)

Various investigations have been conducted with laboratory animals which demonstrate inheritance of such qualities as the capacity to produce antibodies a predisposition to anaphylactic effects and resistance to infection Inbred families of guinea pigs were studied by Lewis *et al* and found to differ as regards ability to engender antibodies (17) and in resistance to tuberculous infection (18) Furthermore Lurie (19 20) has found

[1] A genetic analysis is offered in a paper by Wiener *et al* (11)

in inbred strains of rabbits marked differences towards infection by tubercle bacilli, and he was able to demonstrate several somatic characteristics associated with resistance or susceptibility Well known are the extensive studies by Webster (21, 22), which have resulted in the establishment of strains of mice of high and low susceptibilities to bacterial infections, and to certain viruses

An interesting report dealing with individual differences among guinea pigs in the amount of diphtheria toxin necessary for antitoxic immunization has been presented by Prigge (23) while the ratio of immunizing dose between the extremes among guinea pigs purchased for the Frankfort Institute had the surprisingly high value of 1 32,000, the individual differences with inbred strains, on the other hand, were less pronounced, being only 1 25 for one of these

EXPERIMENTAL

Selection of Parents—Male and female albino guinea pigs were sensitized and tested with 2 4 dinitrochlorobenzene[2] as described below, in order to obtain high and low reactors with which to set up breeding colonies for study of the inheritance of the susceptibility to sensitization in the progeny The animals for starting both the colonies originated chiefly from two sources, one being the breeding room at the Institute, with a few added later from a third stock There were several complicating factors in the choice of initial breeders A selection among low reactor animals is limited because of the scarcity in common stocks of albino guinea pigs refractory to sensitization with dinitrochlorobenzene, a potent sensitizer (24), this is discussed further on Then female guinea pigs exhibit in general a lower level of reactivity than males, making uncertain the estimation of female reactors of the lowest grade Finally, a rather long sensitization procedure may succeed in disguising mediocrity by raising the sensitivity to an apparently acceptable level Largely for these reasons it was found necessary, in addition to selecting the individuals on the basis of their own attained sensitization, to retain them in the breeding colony only after a trial mating and determination of the sensitization level of the offspring Examples are given in Fig 1 (The offspring of unsuitable breeders were likewise excluded) This procedure once established, it proved feasible to erect colonies in which reactivities of the offspring were usually predictable (see Figs 2 to 4)

To start the high reactor colony, 3 males (selected from 25 sensitized guinea pigs) and 6 females (chosen from 20) were employed, on the basis of the progeny test one male and one female were accepted The data for this selection are shown in part in Fig 1, *A*, *B*, *C* Among the six litters resulting when 3 females (Nos 481, 5, 8) were mated in turn with the same 2 males (Nos 343 and 34), there was considerable variability as regards capacity for sensitization, ranging all the way from resistant to highly susceptible litters The choice of Nos 34 and 8 (Fig 1, *C*) as initial breeders appeared expedient Their 3 sons (the mother being no longer available) were bred with a new selection of females (8 picked from 63 sensitized individuals of which 5 were retained after an initial trial mating, and 2, themselves high reactors, transferred from the low reactor colony) By this means and through suitable matings involving 19 females and 21 males produced in the colony (in part back-crosses of offspring to parent and brother sister inbreeding),

[2] Some animals used in the first matings had been sensitized with 2 4 6 trinitrochlorobenzene (picryl chloride) instead

there were secured and tested 112 descendants representing 4 generations after which pen inbreeding using tested males was introduced Some of the matings with less closely related males were made during the several months necessary for the maturing and testing of particular litters

For the low reactor colony similar methods were followed (*cf* Fig 1, *D* and *E*), but consideration was given to the result of the longer course of injections in the progeny as

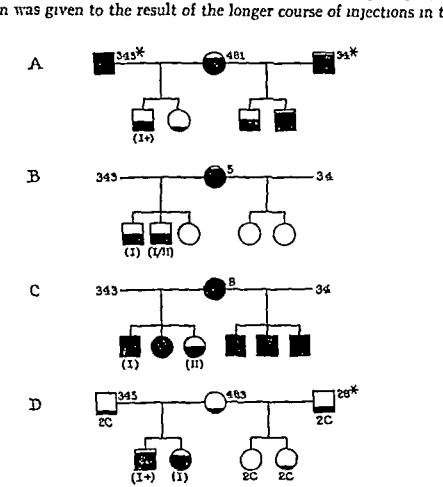

FIG 1 Typical selection for breeding stock among sensitized animals by means of progeny test. Of the parents, those marked with an asterisk were sensitized with picryl chloride instead of dinitrochlorobenzene All offspring were tested with the latter substance

The degree of skin reactivity is indicated by the extent of shading described on pages 714–715, circles and squares designate females and males respectively The symbols show the level of sensitivity after the brief course of treatment (except that 2C signifies test after 14 injections), the sensitivity levels after the second course when determined, is indicated by Roman numerals

Experiences with high reacting animals are given in *A B* and *C*, while trial matings among the low reactors are shown in *D* and *E*

well, exemplified by the choice of Nos 483 and 28 (Fig 1, *D*) and the rejection of Nos 345 ♂, 68 ♀, and 70 ♀ 5 males (picked from 52) and 18 females (chosen from 83) were introduced, 3 of the males and 10 of the females were kept after trial *via* the progeny test From these there have been 110 descendants, representing 4 family generations, produced by the initial matings and by the breeding of 17 female and 10 male offspring

Maintenance of the Colonies—The animals were kept uniformly on a "dry diet," receiving hay, oats, and liberal amounts of chopped cabbage daily Not more than 4 sows were housed with one buck, the females were removed to individual cages in the 2nd month of pregnancy, and offspring were segregated by sexes at an age of about 4 weeks The guinea pigs remained in good health, without intercurrent infections[3] Incidentally animals in the colony, skin-tested with Moen's antigen to detect carriers of streptococci causing epizootic lymphadenitis (25), were found not to react

Sensitization Procedures—Testing of the progeny was commenced when the animals weighed between 340 and 400 gm , at the most 500 gm , the females were not mated before the final test results were obtained Adequate numbers of male and female offspring from both the high and low reactor colonies were assembled for each sensitizing course, thus ensuring comparative testing, and cancelling possible seasonal or other variations due to external influences on the selection of animals for breeding

For sensitizing, injections of 1/400 mg dinitrochlorobenzene in 0 1 cc saline were made into the skin of the back, solutions of the proper concentration were prepared freshly each time by diluting in saline an alcoholic 0 3 per cent solution of the recrystallized commercial preparation It was soon learned that fewer injections of the incitant were necessary to sensitize animals of the "high reactor" class All progeny therefore were first given 4 injections, twice weekly for 2 weeks, and were tested 2 or 3 weeks after the last injection this first, brief course assisted the establishment of the "good" colony by indicating the superior reactors Next the animals received a further course of 10 daily injections, and were tested again 3 weeks after the final injection This longer treatment served principally to detect animals of intermediate capacity for sensitization and to exclude them from the low reactor colony,[4] at times the reactivity was found to have declined somewhat following the longer treatment (7 out of 66 animals in the susceptible colony, 13 out of 88 in the resistant colony)

For testing the sensitivity, 1 drop of a 1 per cent solution of 2 4 dinitrochlorobenzene in olive oil was spread on the belly, after clipping the hair, over an area of 8 to 10 sq cm , fresh sites were used for each test and normal animals were included as controls The reactions were examined on the next day, following use of a depilatory 2 or 3 hours before All males in an experiment, and likewise the females separately, were put together and then sorted out comparatively into four primary classes of reactors (negative up to high reactors) and the ratings were recorded without knowledge of their origins The intensity of the reactions and the assigned grades were as follows pink, commonly slightly elevated (I), pale pink (II), faint pink (III), negative or at most a minimal reaction (IV) Intergrades, as I/II, also were definitely recognizable The symbols in Figs 1 to 4 indicate the gradings by the relative amount of shading, I being shown as

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[3] An exception was the loss of 261 ♂, 262 ♀, in the more resistant colony

[4] Likewise Webster (21, 22) originally used different doses of living bacteria in the establishment of his susceptible and resistant mice

entirely black IV as white II/III as half black and so on A special symbol (see Figs 2 4) is assigned to unusually superior reactors which may be known as I+, namely those showing bright pink, often somewhat swollen test sites Readings after the first and second courses are indicated by an arrow *e g* II → I

Since information was desired about the responses of the guinea pigs in the colonies to a second type of incitant some of the lots following the terminal dinitrochlorobenzene testing were sensitized to poison ivy (26, 27 *cf* 28) using poison ivy extract (Lederle) [5] a 13 per cent solution in acetone of extractives from *Rhus toxicodendron radicans* To effect sensitization, 1 drop of a 1 5 dilution in alcohol was allowed to fall on the lumbar region of the back and was spread with a glass rod over an area about 15 mm in diameter On the 4th day the ivy was removed by cotton pledgets soaked in acetone The test for cutaneous sensitivity, described in detail elsewhere (28) was made between the 10th and 14th days single drops of dilutions in alcohol being applied to various areas of the skin The reactions were recorded at 24 and 48 hours, and ratings assigned according to the lowest effective dilution and the intensity of the reactions (These methods were used also in some breeding experiments undertaken with respect to susceptibility towards ivy sensitization)

As regards the selection for high reacting animals, the responses of the progeny to sensitization with dinitrochlorobenzene are presented in Fig 2[6] and are set forth summarily in Table I The symbols indicate the skin reactivities developed by the "brief" course of 4 injections, with only 0 01 mg in all of the incitant It may be remarked at the outset that skin tests on some of the young made within 10 days after birth or when 2 to 3 months old were negative, proving that we were not here dealing with direct acquisition of hypersensitivity[7] from the sensitized mothers, furthermore, the onset of skin sensitivity to the successive injections has not been different in these animals from that observed with bought guinea pigs Of the males born in the colony, 68 per cent became reactors of grade I following the brief course, and by further treatment with the incitant the proportion of animals in grade I was increased to some extent, *e g* from 66 6 to 78 per cent in the 36 so treated (Table I)

[5] This was supplied through the courtesy of Dr Arthur F Coca

[6] For graphical reasons there have been omitted from Fig 2 the following crosses 387 ♂ × 378 ♀ giving a daughter (II → I), the latter × father 387 giving one I + son, and one son and one daughter of grade I → I + 681 ♂ × 197 ♀ producing 556 ♂ (I) and a daughter of grade II and 556 ♂ × mother 197 giving a daughter of grade III → II 387 ♂ × 197 ♀ producing a daughter (I/II → I) There were two instances of fertilization due to error in the case of 94 ♀ impregnation was known to have occurred in a cage containing 3 males all of grade I

[7] This is also evidenced by the general correlation between the degrees of hypersensitivity induced by dinitrochlorobenzene and poison ivy extract discussed further on which was seen in animals whose ancestors had not been treated with ivy extract

In detail, among these the retest after the second course gave the following result with 13 there was no change in classification, slight changes, as I/II → I, occurred with 14, there was a substantial increase in the grading of 6, and with 3 the early high sensitivity (I) had declined to II, II, and III respectively

Of the 45 females, 42 2 per cent were of grade I after the short treatment, and following another 10 injections in 30 of the animals there was a pro-

TABLE I

Sensitivity Responses of Progeny in the Susceptible and the Low Reactor (More Resistant) Colonies

Grade of response	Susceptible colony				Low reactor colony			
	Animals given both courses		Brief course only	Long course only	Animals given both courses		Brief course only	Long course only
	Brief course	Long course			Brief course	Long course		
	Males							
I+	5	7	6			1		
I	19	21	15	1	2	6		
I/II	3	5	2		1	5		
II	3	2	4		2	3		1
II/III	4				4	11	3	
III	2	1	1		9	6		1
III/IV					7	2		
IV			2		16	7	7	
	Females							
I+	1	8	4			6		
I	10	14	4		1	5		
I/II	2	2	2		2	1		
II	6	2	2		2	8		
II/III	3	2	1		5	4	2	
III	4		1		9	8	3	
III/IV	1		1		9	4	1	2
IV	3	2			19	11	1	1

nounced general rise in sensitivity, the proportion in grade I increasing from 36 6 to 73 3 per cent, and in effect obliterating the sex difference

Due to limited options, probably connected with the lower susceptibility of females, it proved necessary to use as breeders a number of females which were not especially superior after the brief course while 18 out of the 21 bucks were early grade I reactors, this was true of only 14 out of 27 females It may be supposed, and inspection of the applicable data would suggest, that an adequate choice among females would have improved the status of the colony For this purpose, the mothers and their offspring are assigned to three classes in the appended tabulation on the basis of response to the brief sensitization course, uniformity among the fathers as regards early high sensitization appearing to permit an approximation of this sort

Early rating of sows	No of sows	Classification of offspring following brief course		
		I	I/II	II and below
I	12	25 (9 I+)	2	7
I/II	6	17 (2 I+)	3	11
II and below	8	15 (2 I+)	2	12

It appears that nearly three fourths (73 5 per cent) of the offspring of grade I mothers are themselves of grade I, as compared with about 55 and 52 per cent of the offspring of mothers of the two lower categories respectively And likewise suggestive perhaps, is the proportion of animals exhibiting unusually brilliant reactions (designated as I +) born to the females of early high *versus* those of intermediate reactivity

The genetic evidence of Fig 2, by itself, suggests forcefully the segregation of factors influencing susceptibility to sensitization Apart from the animals in class I, there is a variety of lesser grades, a common type being II/III → I or I/II Familial tendencies in respect to this 'delayed" sensitization are probably shown by 393 ♀ (IV → I), its sister 392 (III/IV → I) and 2 daughters of the latter by different males, 681 ♂ and 253 ♂, the ratings in question being II/III → I and III → I + respectively, and in the extreme case by 304 ♀ (IV → I+) and its three offspring by the grade I reactor 44 ♂ (see page 722) 2 animals given the full 14 injections responded slightly or not at all (268 ♀, III → IV, 370 ♀, IV → IV)

The following instances may be cited in which an inhomogeneous condition in the parents was not revealed fully at least by their phenotype testing but by segregation in the progeny The mating of son and daughter from the backcross of 387 ♂ with the latter s mother these 4 all being of early grade I, gave 2 daughters with unlike reactions, one of grade I +, the other II/III Sharp contrasts among the offspring occurred also from the brother sister cross 363 × 362 (*cf* 360 ♀ born to 681 ♂ × 388 ♀) Again from the backcross of 464 ♀ with its father 3 offspring were raised one male a III the other brother and sister each of grade I thereupon a mating of the high reactor brother (255) with its sister gave rise to a son of grade II/III and 2 grade I females while a backcross to its mother produced 2 males and a female which were II IV, and I respectively Then matings of 97 (I) with its mother 378 (II) gave 5 offspring which fell into several types IV → IV (♀) II/III → I (♂) II → I (2 ♀ ♀), I → I (♂) A similar case of one offspring conspicuously resistant to sensitization in contrast to its brothers and sisters is afforded by the 5 progeny from the brother sister mating 312 (I+) × 314 (I +)

In two families probably because of small numbers only grade I reactors were encountered (44 ♂ × 152 ♀ 46 ♂ × daughter 380)

Surveying the results obtained with the strain of high susceptibility, it has been possible to secure a marked improvement over usual guinea pig stocks After the second generation, the regularity of the response to

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II/III	4				4	11	3	
III	2	1	1		9	6		1
III/IV					7	2		
IV			2		16	7	7	
	Females							
I+	1	8	4			6		
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II	6	2	2		2	8		
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Fig 2
Susceptible colony
(Brief sensitization course)

(The special symbols ■ and ● are used for animals of grade I+)

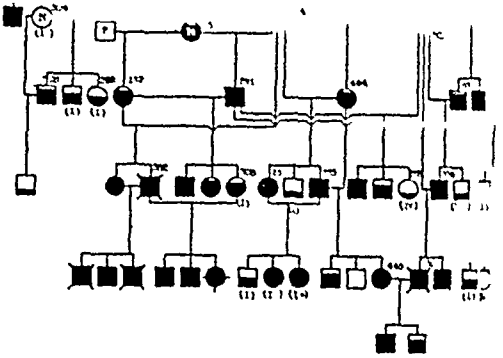


Fig 3
Guinea pigs with low susceptibility
(Brief sensitization course)

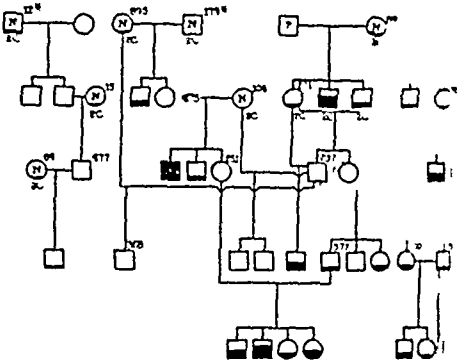
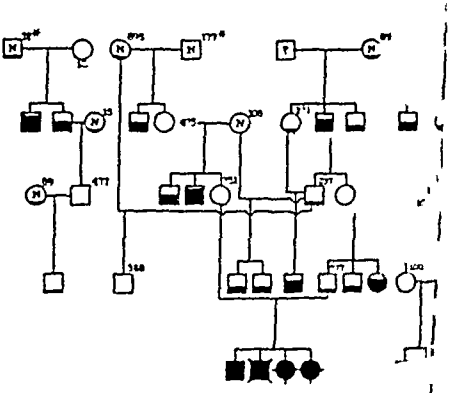


Fig 4
Guinea pigs with low susceptibility
(Both sensitization courses)

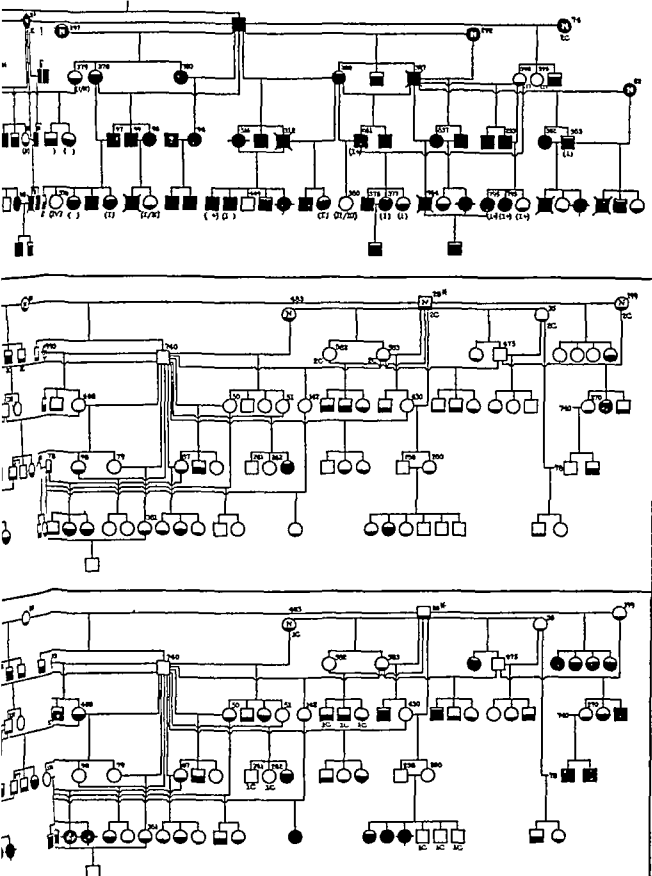


FIGS 2 to 4 The levels of sensitivity are indicated by relative extent of shad... those entirely black (grade I, superior reactors), in addition, the special devices no... respectively

The symbols show the degree of sensitivity attained by the stated procedure, ... courses of injections respectively In Fig 2 the grade after the second course, w...

An identification number without accompanying symbol indicates that the anim...

N = animals introduced into the colonies, some of these (*) had been sensitized ...



ages 714–715), varying from symbols without shading (grade IV, resistant animals) to
bove are used to designate particularly brilliant reactors among the males and females

cept that if data are wanting 1C and 2C signify the result after the first or second
significant is given in Roman numeral subscripts
question appears elsewhere in the figure, usually in the same horizontal line.
picryl chloride

experimental sensitization was not considerably increased by such selective matings as were practicable, the lack of a sufficient number of highly susceptible females probably contributing to this But it would appear that the occurrence of animals exhibiting especially brilliant reactions (I+), often after only the brief course of injections is increasing in frequency with continued selection of parents (Table II) Among the females of the fourth generation for instance, 10 out of 23 were superior reactors at some time during the sensitization, as compared with 2 out of 12 in the third generation Again, in preliminary tests titrations by applying to the skin decreasing concentrations of the incitant have indicated that, whereas animals from unselected stocks sensitized by comparable procedure and picked as good reactors seldom reacted to a 1/25 per cent solution in olive oil, definite

TABLE II

Occurrence of Animals of Especially High Reactivity (Grade I+)

The number of I+ animals is shown first, followed by the total number submitted to the given sensitization procedure *i e*, one or two courses

Generation	Males		Females	
	I+ after brief course	I+ developed by 2d course	I+ after brief course	I+ developed by 2d course
F_2	1/8	0/4	0/9	0/4
F_3	2/20	1/18	1/12	1/10
F_4	8/28	3/11	4/23	6/11

reactions to this concentration have been observed with about two-thirds of the limited numbers of high reactor progeny tested

It may be mentioned that also by another mode of sensitization, namely repeated applications to the skin of alcoholic solutions of the incitant, animals produced in the high reactor colony have shown themselves superior to other stocks

In sharp contrast to the high reactor colony are the results with the strain bred for low susceptibility The reactivity after the brief course of injections is shown in Fig 3[1] and the effects seen after the second course are given separately as Fig 4 The evident disparity between Figs 2 and 3 is not extinguished even by the longer treatment, although the latter nearly always brought about an increase in reactivity From the data in Table I, 41 per cent of the offspring raised in the colony belonged in grade IV after the short course and 3 out of 4 (77 1 per cent) were grade III and below,

[1] The data in Figs 3 and 4 do not show some lines which were abandoned in the third generation because of too few offspring

that is, worthless for most experimental work in drug allergy, there were no significant sex differences When further injections of the incitant were given, only 17 per cent of the males and 23 4 per cent of the females were of grade IV, although 36 6 and 49 per cent respectively were still not higher than grade III It should be added that, while a few guinea pigs in average albino stocks appear to be refractory after 10 to 15 daily injections of 2 4 dinitrochlorobenzene, it is highly doubtful whether an absolute resistance will be encountered in any guinea pig under more intensive treatment, for instance repeated applications of oil or alcohol solutions to the skin [9] The lowest category used in the charts, grade IV, does not signify entire resistance, for some trifling hyperreactivity to the daily injections was noted in nearly all these animals during the second course, however, in the final contact test after the rest period the animals could not be distinguished surely from those of the normal controls which showed some slight skin irritation to the chemical

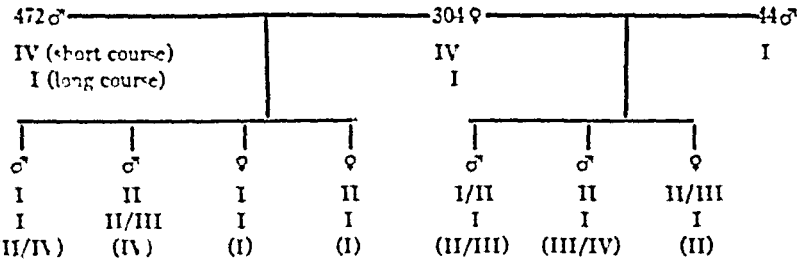
The greater difficulty in breeding for low susceptibility to sensitization was evident in the earliest attempts Contributing complications in the selection of the breeding stock were mentioned above (page 712) probably as a consequence there was less initial homogeneity than among the high reactor stock As regards improvement of the colony, a choice of "continuing low reactors among the progeny, particularly brothers and sisters, was quite limited Of the 7 male offspring suitable for breeding 2 had given slight reactions after the brief course while among 11 females attempts to mate 4 were fruitless the breeding of 2 others was scarcely productive, and the remaining 5 included one which had been early grade III and 2 that were early grade II/III

Since the tendency for resistant animals to produce susceptible offspring is much greater than the reverse, it is probable genetically that the chief factors contributing to resistance are dominant over those favoring susceptibility to experimental sensitization A few instances of "early resistant" animals from the mating of susceptibles (Fig 2, *e g* 449 ♂, and Fig 1, *B*) appear, however, to be out of line with a simple presumption that "resistance is dominant over susceptibility" Indeed, the data otherwise suggest that a plurality of factors controls susceptibility and resistance, in particular because of the occurrence of a rather large number of patterns, instead of a few, in the acquired levels of sensitivity Other than this, if

[9] Of some 234 albino guinea pigs sensitized in this manner by only 5 to 7 daily applications not more than 4 appeared to be refractory This would then be analogous to the situation with *Primula* dermatitis (16), which is acquired by nearly every individual upon intensive treatment with extractives of the plant although under natural conditions of contact marked individual differences in the capacity for sensitization are evident

one considers only the following broad types early susceptible delayed susceptible (as III → I) and continuing resistant (IV → IV) it is not probable that a single pair of allelomorphs is adequate to explain the sensitization behavior of the progeny e g in the line father 28 × 583 ♀, 28 × 430 ♀ brother 258 × 260 ♀ (Figs 3 4) The intricacy is further to be seen from the various matings of 740 ♂ and of 78 ♂ (and from the discrepancies mentioned below between the sensitivities caused by use of two irritants) It will be noted in this colony that throughout the breeding of guinea pigs selected for their relatively high resistance, we did not meet with any positive instance of a "homozygous" animal as demonstrable by the testing of its progeny (although this well could be a consequence of the limited combinations of parents employed)

Of interest in connection with the genetic situation are the results of mating two delayed reactors of unusual behavior, and the female also with an early susceptible male the gradings after the brief and second courses are given, and below, within parentheses the sensitivity developed to poison ivy following treatment of the progeny with the latter



Crosses involving members of the susceptible and resistant colonies were not undertaken since the latter group was not sufficiently uniform

The essential difference between the two colonies and the evident segregations in the offspring indicate a genetic basis for susceptibility to drug allergy in the guinea pig albeit a complicated one, and this is supported by the finding (after our breeding experiments were well along) of distinctly different levels of susceptibility among the albino stocks offered by various breeders Among these instances may be mentioned one stock characterized by a low to moderate susceptibility to sensitization by intracutaneous injections of dinitrochlorobenzene and another which was distinctly inferior to other animals in our experience as regards skin sensitization to picryl chloride following intraperitoneal injection of this irritant in conjunction with dead tubercle bacilli (29)

We also conducted analogous breeding experiments with animals chosen

on the basis of high and low sensitivities developed to poison ivy (see page 715) instead of to dinitrochlorobenzene The number of progeny examined was not large, but the results tended to show that there is an hereditary basis for susceptibility in this case also

The question arose whether the difference between the two colonies is actually one of sensitization capacity or simply a lower or higher resistance to the primary toxicity of the incitant (upon which acquired sensitivities, possibly not unlike in degree, would be superimposed) For this purpose, 4 males and 4 females from each colony were assembled and, prior to the regular sensitization course, the skin reactions (primary toxicity) to different concentrations of dinitrochlorobenzene (1 5 to 0 75 per cent in alcohol, 10 to 1 per cent in olive oil) were determined The brief intracutaneous sensitization course was then given, and after a rest period of 2 weeks the animals were tested with a 1 per cent solution in olive oil (which is seldom irritating and which had not sufficed to differentiate any of these same animals before sensitization)

There was, in fact, a difference between members of the two colonies, particularly among the females, with respect to the primary toxicity of dinitrochlorobenzene, skin irritations being produced in members of the high reactor colony by about one half to one third the concentration giving rise to the same degree of irritation in the low reactor colony The significance of this as an explanation of the difference between the two colonies apparently can be discounted, however, because there was no consistent parallelism in the individual cases between primary toxicity and the level of hypersensitivity attained by treatment with the incitant For instance, among the males from the high reactor colony the one exhibiting the greatest primary toxicity happened to be a poor reactor, and among the females from the low reactor colony the one least irritated by the incitant developed the highest sensitivity of the group Another potent argument for actual differences in degrees of specific sensitivity is the frequent occurrence in the susceptible group of reactions to 1/25 per cent solutions of the incitant in olive oil, mentioned above, as compared with the poor responses to the 1 per cent test solution among members of the low reactor colony, a ratio much greater than the one between the concentrations producing irritation in non sensitized animals of the two colonies Nevertheless, this somatic difference between members of the two colonies may have a bearing on the multiplicity of sensitization patterns observed

With a number of guinea pigs (100 progeny from the susceptible and resistant colonies, 37 offspring of animals selected according to their response to poison ivy extract, and 27 other guinea pigs), sensitization courses were

given successively with the two incitants to compare the respective responses. There was, in fact, commonly a parallelism between the degrees of sensitivity acquired to the two types of incitant, as stated for human beings by Wedroff and Dolgoff (5), yet discrepancies have appeared. These have been chiefly in the direction of a dinitrochlorobenzene sensitivity higher than that towards ivy, but there were at least 20 clear instances of the reverse.

Experiments of this sort should be extended, perhaps with the breeding of animals having unequal sensitivities to two incitants, and using methods of sensitization chosen to allow simultaneous testing with the respective chemicals.

The relationship observed will depend obviously upon the intensity of treatment with each sensitizer. For instance, the correlation was higher if the response to the ivy extract was compared with the dinitrochlorobenzene brief course. The experiences with 46 animals from the susceptible colony and 54 from the more resistant colony may be cited after the brief course, 52 per cent of the offspring showed close agreement between the responses to the two incitants (*e.g.*, variations of not greater order than II *versus* I/II, IV *versus* III/IV, etc.), and after further dinitrochlorobenzene treatment in the second course 42 per cent were still in close correspondence.

Other data bearing on discrepancies may be cited.[10] 2 males from the DNCl (dinitrochlorobenzene) stock which had scarcely responded to picryl chloride after a course of 13 intracutaneous injections of this substance were then given the brief course of DNCl injections, both became definitely sensitive to the second incitant (I/II and III respectively), and the discrepancy was confirmed upon retesting with picryl chloride and dinitrochlorobenzene simultaneously. Again, in one experiment 20 guinea pigs from common stocks were sensitized concurrently with both ivy and dinitrochlorobenzene, the latter being here used as a 5 per cent solution in olive oil applied to the skin daily for 4 days, and were tested with the two substances at the same time. The sensitization levels with the two incitants corresponded in 15 animals, of the others, one was moderately sensitive to DNCl but quite high with ivy while two others showed the opposite relationship, another was high towards DNCl and very real towards ivy, and still another exhibited rather good DNCl and low to moderate ivy responses.

COMMENT

The establishment by controlled breeding of two colonies of guinea pigs which differ significantly in the degree of sensitivity attained following the same sensitizing procedure demonstrates the existence of variations of an hereditary nature in the capacity for sensitization. The difference between the two colonies was striking in that the one strain gave in the great majority

[10] Compare the ivy and dinitrochlorobenzene responses in the tabulation on page 722.

of cases uniformly intense reactions after a brief course of intracutaneous injections with a total of 0 01 mg substance, while the other responded almost regularly to an even greater number of injections with only a low grade sensitivity, however, these animals were not entirely refractory In the latter group the uniformity was much less pronounced than among the good reactors, that is to say the offspring of poor reactors not seldom proved to be unequal, some individuals in a litter frequently exhibiting stronger effects than either parent It might well be, however, that continuation of selective inbreeding would eventually lead to a more uniform strain of poorly reacting animals It may be remarked that also Webster in the studies referred to above had greater difficulty in establishing a strain of mice with high resistance to infection with mouse typhoid than a highly susceptible strain

Suggestive evidence for inherited differences as regards drug sensitization comes forth from experiences with guinea pig strains procured from different breeders It appeared that guinea pigs (albinos) from some sources re sponded so poorly to sensitization that they were unsuitable for our experimental purposes, even after being kept for some time under our regimen

The experiments were not carried far enough, and the situation is as yet too complicated, to offer a genetic analysis Also it was not feasible to undertake repeated matings between parents of different types to obtain information about the ratios in the offspring Several features, particularly the fact that the sensitivities do not fall into a few sharply discrete grades but show continuous transitions, would appear to contravene a supposi tion of a single pair of genetic factors

Breeding experiments with parents selected for their reactivity to poison ivy, although made only on a small scale, tend to show that here again the degree of susceptibility is hereditary and that good and poor colonies can be established This raises the point whether animals of different suscepti bilities to sensitization with one simple substance will show the same behavior towards a different compound, that is, whether one deals with a susceptibility that is general or one varying to some extent according to the substances tested From experiments in which animals were sensitized in succession to dinitrochlorobenzene and poison ivy, it would seem that there is roughly an accordance between the sensitivities developed to the two compounds (*cf* 5) but there were several instances of discrepancy one way or the other If further experience were confirmatory, this likewise would affirm the complexity of the hereditary basis underlying drug allergy Observations indicating a degree of specificity in experimental sensitization of human beings have recently been reported (30)

It may be added that the possession of guinea pigs highly and uniformly susceptible to sensitization should be of value for experimental work in this field

SUMMARY

It has proved possible to set up lines of guinea pigs of significantly different susceptibilities towards a compound of simple structure, namely 2 4 dinitrochlorobenzene This provides direct evidence that the type of sensitization under discussion is influenced by heredity

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CASEIN DIGESTS PARENTERALLY UTILIZED TO FORM BLOOD PLASMA PROTEIN

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The gist of this paper is that certain casein digests given by vein or subcutaneously are promptly used by the hypoproteinemic dog to produce needed plasma proteins. The digest of casein is approximately as effective by vein as is the casein digest or whole liver equivalents given by mouth. The digests tested are essentially non toxic as used.

A considerable series of experiments on dogs done in this laboratory has been reported during the past several years (9, 15, 4, 11) to show that *normal dog plasma* given by vein to the protein fasting dog can supply all *protein requirements*. The dog has been kept many weeks in health, weight balance, and positive nitrogen equilibrium by suitable amounts of plasma by vein and sugar, fat, minerals, and accessories by mouth.

Normal dog plasma protein given by vein during such protein fasts is utilized much more effectively than protein by mouth. It is evident from these previous experiments, and others of Howland and Hawkins (10), that the plasma proteins are utilized in the body more directly, with less waste and with but slight cleavage into large aggregates when needed to supply the protein requirements of the fasting dog. This exchange of proteins between the blood plasma and body cells has been discussed in recent papers (13, 14).

Table 5 (period 17) also shows how effectively plasma protein is used in the fasting dog as compared with the casein digest. Note the low urinary nitrogen when plasma protein is given by vein—about one half the amount recorded when equivalent amounts of casein digest are given by vein. However, the casein digest by vein is as effective in plasma protein formation as liver protein or casein digest given by mouth. This would support the current belief that practically all *food proteins* are reduced to amino acids and peptides before utilization for protein building in the normal body.

In the experiments tabulated below we assume that hypoproteinemia offers a strong continued stimulus to plasma protein regeneration. Daily

plasmapheresis and a constant basal diet enable the investigator to keep the plasma protein concentration close to 4 per cent Maintenance of the plasma protein level near 4 per cent for many weeks is essential for steady maximal stimulation (13) and effects a relatively constant protein output, a product of the basal diet In the early weeks a surplus is removed over and above the basal plasma protein output this surplus is called the *reserve store* and varies with different dogs and different dietary régimes in effect before the plasmapheresis The nature of this reserve store has been the subject of some discussion (14, 3, 2, 13)

By this type of experiment with carefully standardized dogs, we are able to determine *quantitatively* the worth of diet factors and this has been done for many diet proteins The method is admirably suited to a study of protein digests designed for clinical use It is possible to measure accurately the result in plasma protein removed and compare this result with the response to standard proteins by mouth

Protein digests parenterally were tried years ago (8), and more recently Elman (5, 6) and others (16, 7, 1) have reported several favorable nitrogen balance studies with them Elman notes rises in the plasma albumin level, Farr (7) finds no effect on the plasma protein concentration of nephrotic children

Methods

All *dogs* were immunized against distemper They were under close observation in metabolism cages

The *diet materials* were analyzed for nitrogen dried yeast (type 200-B, Standard Brands, Inc.), 7 88 per cent, liver powder (H 8083, Eli Lilly and Company), 9 66 per cent, commercial casein, 14 0 per cent, fresh pork liver, 3 20 per cent Their protein content was assumed to be 6 25 times their nitrogen value The salt mixture of Wesson (17) was used The commercial lecithin contained 3 53 per cent choline

We are aware that the vitamin supply of the oral diets, given as listed in the Experimental Histories, may not prove entirely adequate when more is known about the requirements of the individual members of the B complex, particularly in animals under the strain of plasmapheresis over long periods of time To add more crude sources, such as yeast is to complicate further the finer interpretations of the results of protein testing We emphasize, however, the excellent clinical condition of these dogs

The *casein digest L* used in these experiments was furnished us through the courtesy of the Research Laboratories of Eli Lilly and Company We are particularly indebted to Mr Elmer Stuart, Mr George B Walden, and Dr G H A Clowes The material is prepared by papain digestion of commercial casein The digestion goes on in an acid medium at a temperature of approximately 40° during several days Final digest mixture is heated to boiling, cooled, filtered, and the filtrate evaporated to dryness in a vacuum A golden yellow, granular material is the end product It has a nitrogen content of 12 5 per cent and an assumed protein equivalence of 78 1 per cent

The *protein digest X*, the product of another laboratory, is said to be a combination of acid and alkali hydrolysates of protein, brought into a final solution containing 1 per cent nitrogen and 5 per cent dextrose Its assumed protein equivalence is 6 25 gm per 100 cc. solution

The *procedures* used have been previously described for the most part (12) Aseptic precautions in the plasmapheresis technique and in the infusion of digest solutions were not taken in obtaining the data of Tables 4 and 5 nor in the first 8 periods of Tables 1 and 3 Aseptic methods were used elsewhere Casein digest L and dextrose were brought into solution in previously boiled hot water and then filtered through a Seitz (EK) filter pad, with a final concentration of 5 per cent each Dextrose was not added to the digest in the subcutaneous injection tests of Table 2 The rate of injection on different occasions varied from 4 to 8 cc. of the 5 per cent solution per minute No change in the urinary nitrogen was associated with a change in rate within these limits The daily quota of digest was given in 2 equal injections about 5 hours apart except as noted in Tables 1 and 1 a and in Clinical History, dog 39 106 When plasmapheresis was performed the first digest injection would immediately follow the return of the washed red blood cells The oral diet was offered about 2 hours after the last digest injection for the day When all the digest was given in one injection, the oral diet was offered about 6 hours later

We realize that for maximum utilization the digest infusions should *follow* the ingestion of the non protein portion of the meal of the day They are practical objections to such a procedure Moreover, no certain difference in utilization was noted when the second half of the digest and the diet were given only 2 hours apart (Tables 1 and 1-*a*)

EXPERIMENTAL OBSERVATIONS

The following five double tables include data obtained from testing protein digests in 5 dogs In Tables 1 and 1 *a*, 2 and 2 *a*, 3 and 3 *a*, the casein digest L was given to dogs rendered hypoproteinemic by plasmapheresis The same digest was given to 2 normal dogs as reported in Table 5 The protein digest X was given to a dog during plasmapheresis in Tables 4 and 4-*a* and to the 2 normal dogs of Table 5

Casein digest L, given by vein or subcutaneously, is well utilized for plasma protein formation and for maintenance of nitrogen balance during hypoproteinemia It promotes recovery from hypoproteinemia (Tables 1 and 2) It is utilized in normal dogs not subjected to plasmapheresis (Table 5) but insufficiently well to achieve nitrogen balance This casein digest L appears to be just as effective in plasma protein regeneration when given by vein as when given by mouth (Table 3, periods 7 and 8), despite the fact that by vein it is always associated with an increase in urinary nitrogen, in other than the urea and ammonia fraction (Tables 1 *a*, 2 *a*, 3 *a*, and 5) This urinary nitrogen increase remains essentially the same and the utilization appears the same when the digest is given in one continuous dose rather than in 2 doses hours apart (Tables 1 and 1 *a*) The

utilization of this digest is not improved by the addition of tryptophane or of cystine or of both these amino acids (Tables 1, 3, and 5)

The *protein digest* V if supplemented by cystine and tryptophane is well utilized in plasma protein production and induces a positive nitrogen balance

TABLE 1

Casein Digest I Promotes Plasma Protein Production
Cysteine and Tryptophane Not Required as Supplements

Dog 39-223

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Blood plasma Average concentration		RBC hematocrit average	Plasma volume	Weight
				Total protein	A/G ratio			
		gm	*gm*	*per cent*		*per cent*	*cc*	*kg*
	Kennel	—	—	5 92	1 26	40 9	366	8 9
1	Fasting	0	25 4	5 44	1 28	41 6	292	7 9
2	Low protein	15	14 9	3 95	96	48 3	312	8 0
3	Liver basal	85	20 9	4 12	1 02	48 5	300	8 3
4	Liver basal	85	21 9	3 95	1 11	47 4	316	8 5
5	Liver basal	85	21 0	4 07	1 10	46 3	328	8 5
6	Liver basal	73	21 0	4 12	1 08	49 2	253	8 5
7	Low protein + *digest L* + *tryptophane* + *cysteine**	76	19 4	4 04	0 91	49 8	322	8 5
8	Low protein + *digest L* + *cysteine*	81	17 4	3 93	0 79	47 4	271	8 8
9	Low protein + *digest L*	79	22 8	4 27	0 68	45 0	—	8 5
10	Low protein + *digest L*	81	18 6	3 98	0 66	46 5	—	8 5
11	Low protein + *digest I*	81	3 4	4 54	0 69	46 1	—	8 7
12	Low protein + *digest I*	81	3 3	5 08	0 76	42 1	368	8 7
13	Low protein + *digest L*	81	1 6	5 18	0 76	43 5	368	8 9
14	Low protein + *digest L*†	81	1 7	5 29	0 83	46 0	—	9 0
15	Low protein + *digest I*†	81	3 3	5 56	0 90	46 9	—	9 2
16	Low protein + *digest L*†	81	1 8	5 59	0 87	45 8	—	9 2
17	Low protein + *digest L*†	81	23 5	4 54	0 83	54 8	—	9 4
18	Low protein + *digest L*†	77	17 6	4 07	0 75	52 2	—	9 3
19	Low protein + *digest L*†	76	12 9	4 04	0 69	45 6	—	9 3

* Items in italics given intravenously

† Digest given in 1 injection daily

(Tables 4 and 4-*a*) In striking contrast, if these amino acids are omitted, the plasma protein output drops and the urinary nitrogen excretion shoots up

Experimental History.—Dog 39 233 (Tables 1 and 1-*a*) An adult female terrier, fasted during period 1, was given during period 2 a low protein diet consisting of cane sugar 95 gm., corn starch 20 gm., corn oil 10 gm., crisco 10 gm., salt mixture 5 gm.,

bone ash 10 gm , cod liver oil 5 gm dried yeast 2 gm liver powder 2 gm , nicotinic acid 0 05 gm , choline hydrochloride 0 4 gm

TABLE 1-*a*

Nitrogen Balance

Casein Digest L Well Utilized

Strongly Positive Nitrogen Balance after First Two Periods

Dog 39 223

Period 7 days	Diet	Nitrogen balance							
		Intake		Output					Intake minus output
		in diet	in excess R.B.C. injected	in plasma	in feces	in urine			
						Total	Urea + NH_3	Undetermined	
		gm	*gm*	*gm*	*gm*	*gm*	*per cent*	*gm*	*gm*
	Kennel	—	—	—	—	—	—	—	—
1	Fasting	0 0	3 6	4 2	—	12 6	—	—	−13 2
2	Low protein	2 4	0 1	2 5	‡	4 9	—	—	−4 9
3	Liver basal	13 6	2 0	3 4	3 5	8 8	—	—	−0 1
4	Liver basal	13 6	0 3	3 6	1 9	8 3	—	—	+0 1
5	Liver basal	13 6	0 1	3 4	2 2	7 8	—	—	+0 3
6	Liver basal	11 6	0 9	3 4	1 7	7 4	71	2 2	0 0
7	Low protein + *digest L* + *tryptophane* + *cysteine**	12 8	0 7	3 2	2 0	10 2	57	4 4	−1 9
8	Low protein + *digest L* + *cysteine*	13 4	1 7	2 8	1 9	10 4	56	4 6	0 0
9	Low protein + *digest L*	12 5	2 7	3 4	1 6	11 1	58	4 7	−0 9
10	Low protein + *digest L*	12 9	4 0	3 0	1 7	9 9	59	4 1	+2 3
11	Low protein + *digest L*	12 9	1 0	0 6	1 6	9 4	55	4 2	+2 3
12	Low protein + *digest L*	12 9	1 3	0 5	1 6	9 5	56	4 2	+2 6
13	Low protein + *digest L*	12 9	0 8	0 3	1 6	9 6	55	4 3	+2 2
14	Low protein + *digest L*†	12 9	0 8	0 3	1 8	10 1	54	4 6	+1 5
15	Low protein + *digest L*†	12 9	−0 5	0 5	1 8	9 9	51	4 8	+0 2
16	Low protein + *digest L*†	12 9	−1 5	0 3	1 7	9 4	51	4 6	0 0
17	Low protein + *digest L*†	12 9	5 3	3 8	1 8	9 7	54	4 5	+2 9
18	Low protein + *digest L*†	12 3	2 6	2 9	1 1	10 0	54	4 5	+0 9
19	Low protein + *digest L*†	12 2	2 4	2 1	0 9	9 2	55	4 2	+2 4
	Totals	221 2	28 3	44 2	30 4	178 2	—	—	−3 3

‡ Included in period 3

* Items in italics given intravenously

† Digest given in 1 injection daily

In periods 3 to 6 the liver basal diet consisted of the above diet with the addition of raw pork liver, 50 gm , and the deduction of cane sugar 15 gm One day's diet was omitted during period 6

In periods 7 to 19 the low protein diet was the same as that of period 2 except for the addition of thiamin chloride 5 mg and the deduction of cane sugar, 25 gm During

Experimental History.—Dog 39 234 (Tables 3 and 3 a). An adult female mongrel dachshund fasted during period 1, was given during period 2 a low protein diet consisting of cane sugar 110 gm., corn starch 20 gm., corn oil 10 gm., crisco 15 gm., salt mixture 5 gm., bone ash 10 gm., cod liver oil 5 gm., dried yeast 2 gm., liver powder 2 gm., nicotinic acid 0.05 gm., choline hydrochloride 0.4 gm.

During periods 3 to 6 the liver basal diet consisted of the above diet with the addition of raw pork liver, 50 gm., and the deduction of cane sugar, 15 gm. One day's diet was omitted during period 6.

During periods 7 to 9 the low protein diet was the same as that of period 2, except for the addition of thiamin chloride, 5 mg., and the deduction of cane sugar, 25 gm.

TABLE 2

Casein Digest L Subcutaneously as well as by Vein Promotes Plasma Protein Production

Dog 39 316

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Blood plasma Average concentration		R.B.C. hematocrit, average	Plasma volume	Weight
				Total protein	A/G ratio			
		gm.	*gm.*	*per cent*		*per cent*	*cc.*	*kg.*
	Kennel	—	—	5.35	1.43	56.1	425	10.7
1	Fasting	0	25.6	4.85	1.53	52.1	—	9.7
2	Low protein	15	15.7	3.68	1.37	15.7	390	10.5
3	Low protein + *digest L**	93	12.5	4.14	0.58	46.2	—	9.9
4	Low protein + *digest L*	88	26.1	4.60	0.57	46.1	—	9.7
5	Low protein + *digest L*	95	18.9	4.34	0.57	45.6	—	9.8
6	Low protein + *digest L*	97	15.6	4.04	0.60	48.3	—	9.6
7	Low protein	15	1.5	4.36	0.59	16.0	—	9.5
8	Low protein + digest Lt	97	1.5	4.59	0.68	41.7	—	9.5
9	Low protein + digest Lt	153	2.9	5.42	0.68	12.6	—	9.4

* Items in italics given intravenously.

† Digest given subcutaneously.

Casein digest, 12 gm., *l*-cysteine hydrochloride, 0.8 gm., and *l* tryptophane, 0.25 gm., were injected daily by vein in period 7 and consumption of the low protein diet was 99 per cent, except on the first day of this period. On this day the digest (6 gm.) was given as a 10 per cent solution, salivation was marked, hyperpnea was moderate, and vomiting of watery fluid occurred 3 times during the 30 minute injection. The second 6 gm. of digest were not given. On this day activity was subdued but the dog ate the low protein diet 100 per cent. For the remaining 6 days of period 7, the digest was given as a 5 per cent solution with the same reactions in the dog on each injection but of very mild nature. Vomiting occurred once with 9 of the 12 injections. The reactions were transient and a few minutes after the injection the activity returned to normal.

In period 8, casein digest, 12 gm., *l*-cysteine hydrochloride 0.8 gm., and the low protein diet were offered daily by mouth and 97 per cent consumed.

In period 9 casein digest 12 gm. and the low protein diet were offered by mouth

and the average daily consumption was only 77 per cent The dog appeared in good condition but throughout the period showed a trace of albumin in the urine and rare red blood cells The jugular veins were so scarred by this time that adequate bleedings were impossible and by the end of the period the plasma protein concentration had reached 5 27 gm per cent

TABLE 2-*a*

Nitrogen Balance

Nitrogen Equilibrium Maintained after First Two Periods

Dog 39 316

Period 7 days	Diet	Nitrogen balance							
		Intake		Output					Intake minus output
		in diet	In excess R.B.C. injected	in plasma	in feces	in urine			
						Total	Urea + NH_3	Undetermined	
		gm	*gm.*	*gm.*	*gm*	*gm*	*per cent*	*gm*	*gm*
	Kennel	—	—	—	—	—	—	—	—
1	Fasting	0 0	−2 1	4 2	—	13 3	83	2 2	−19 6
2	Low protein	2 4	2 5	2 6	2 2	10 1	72	2 8	−10 0
3	Low protein + *digest L**	14 8	1 8	2 1	1 8	14 4	63	5 4	−1 7
4	Low protein + *digest L*	14 0	4 7	4 2	1 7	14 3	61	5 6	−1 5
5	Low protein + *digest L*	15 1	2 9	3 1	1 7	12 7	55	5 7	+0 5
6	Low protein + *digest L*	15 5	3 7	2 5	1 4	13 0	59	5 3	+2 3
7	Low protein	2 4	0 7	0 2	1 5	7 1	66	2 4	−5 7
8	Low protein + digest L†	15 5	1 3	0 2	2 1	12 8	64	4 6	+1 7
9	Low protein + digest L†	24 6	−0 5	0 5	1 2	18 1	66	6 2	+4 3
	Totals	104 3	15 0	19 6	13 6	115 8	—	—	−29 7

* Items in italics given intravenously

† Digest given subcutaneously in 2 equal injections daily

The *casein digest L* is as effective in plasma protein production by vein as by mouth (Table 3) despite the fact that more of its nitrogen is eliminated in the urine when given by vein The reduced output of plasma protein in period 9, Table 3, is explained in the Experimental History The negative nitrogen balance of period 9 is largely the result of difficulty in reinjecting red blood cells

Experimental History—Dog 39 106 (Tables 4 and 4-*a*) An adult male beagle hound fasted during period 1 was given during period 2 a low protein diet consisting of cane sugar 70 gm , lard 30 gm butter fat 20 gm cod liver oil 5 gm , lecithin 5 gm dried yeast 1 gm , nicotinic acid 0 025 gm salt mixture 4 gm , bone ash 10 gm In period 3 liver 50 gm , replaced sugar 20 gm , of the above diet and in periods 4 and 5 casein 15 gm replaced the liver These diets were completely eaten every day

In periods 6 and 7, the protein digest X was given intravenously, 200 cc daily, in 4 equal portions, 2 to 3 hours apart, at a rate of 2 5 to 3 cc per minute, without reaction The low protein diet, as described above except for reduction in sugar to 40 gm , was completely consumed in period 6 but in period 7 the appetite and the activity of the dog decreased and a total of 50 per cent of one day's diet was rejected The diet was changed during the last 4 days of the period by adding canned tomatoes, 50 gm , and by increasing the sugar to 100 gm and decreasing the lard to 20 gm , the butter to 10 gm

In period 8, protein digest X, 200 cc , was given daily mixed with the low protein diet of the 6th period and supplemented by *l*-cysteine, 0 8 gm , and *l*-tryptophane, 0 3 gm

TABLE 3

Casein Digest L by Mouth or by Vein

Shows Same Plasma Protein Production as Liver Protein by Mouth

Dog 39-234

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Blood plasma Average concentration		R.B C. hema tocrit average	Plasma volume	Weight
				Total pro tein	A/G ratio			
		gm	*gm*	*per cent*		*per cent*	*cc*	*kg*
	Kennel	—	—	5 87	1 23	43 2	438	10 7
1	Fasting	0	31 8	5 22	1 46	45 5	296	9 5
2	Low protein	15	15 3	3 85	0 95	48 3	317	9 2
3	Liver basal	85	22 1	3 99	0 76	49 4	366	9 3
4	Liver basal	85	20 8	3 93	0 71	47 2	381	9 3
5	Liver basal	85	20 6	4 17	0 78	45 7	384	9 4
6	Liver basal	73	22 0	4 10	0 82	48 4	364	9 3
7	Low protein + *digest L + tryptophane + cysteine**	76	20 3	4 12	0 78	46 8	—	9 2
8	Low protein + digest L + cysteine by mouth	81	19 6	4 07	0 64	46 0	372	9 5
9	Low protein + digest L by mouth	62	10 7	4 48	0 53	44 5	—	9 1

* Items in italics given intravenously

The mixture was completely consumed For the final 2 days of the period tomatoes, 50 gm , and thiamin chloride, 5 mg , were added to the diet

In period 9, casein, 15 gm , sugar, 10 gm , and B_2-liver concentrate, 1 gm , were added to the protein-low diet as of period 6

In the 10th period the diet casein of the preceding period was replaced by protein digest X by vein as in periods 6 and 7 plus the daily addition to the digest of *l*-cysteine hydrochloride, 1 04 gm , and *l*-tryptophane, 0 3 gm The digest and supplements were given on only the last 6 of the 7-day period Consumption of the low protein diet was complete except for 20 per cent on the 6th day and 93 per cent on the 7th day There was no obvious explanation for this decline in appetite although one soon developed The dog appeared generally normal except for slight reduction in activity

Throughout this experiment, including the beginning of the 10th week, the urine

was normal in the gross and microscopically, but at the end of period 10 the urine contained a slight amount of albumin, a few white blood cells and red blood cells and occasional granular casts In the 11th week appetite further declined the dog appeared definitely sick after a few days and was sacrificed one week after the close of the 10th period Blood culture the day before death yielded *Streptococcus viridans* and *B alcaligenes*, and at autopsy similar organisms were recovered from fresh vegetations on the mitral valve Also found were an acute focal embolic nephritis, infected infarcts of

TABLE 3-*a*

Nitrogen Balance

Nitrogen Balance Maintained after First Two Periods

Dog 39 234

Period 7 days	Diet	Nitrogen balance							
		Intake		Output					Intake minus output
						in urine			
		in diet	in excess R.B.C. injected	in plasma	in feces	Total	Urea + NH_3	Undetermined	
		gm.	gm.	gm.	gm.	gm.	per cent	gm.	gm.
	Kennel	—	—	—	—	—	—	—	—
1	Fasting	0 0	6 1	5 1	—	17 2	—	—	−16 2
2	Low protein	2 4	0 8	2 5	3 0	12 3	—	—	−14 6
3	Liver basal	13 6	1 6	3 6	2 6	10 7	—	—	−1 7
4	Liver basal	13 6	0 2	3 4	3 4	9 1	—	—	−2 1
5	Liver basal	13 6	−0 8	3 4	2 5	8 8	—	—	−1 9
6	Liver basal	11 6	1 3	3 6	2 6	9 1	—	—	−2 4
7	Low protein + *digest L + tryptophane + cysteine**	12 8	1 6	3 2	2 3	9 7	59	4 0	−0 8
8	Low protein + digest L + cysteine by mouth	13 3	3 6	3 2	2 7	8 1	70	2 4	+2 9
9	Low protein + digest L by mouth	10 0	−5 8	1 8	2 0	8 3	72	2 3	−7 9
	Totals	90 9	8 6	29 8	21 1	93 3	—	—	−44 7

* Items in italics given intravenously

heart, spleen, and kidneys, hemorrhages in serous membranes and gastro-intestinal tract. From histological examination the apparent age of the vegetations agrees with the clinical onset of the illness about 10 days before death, no doubt a consequence of the multiple intravenous injections without aseptic precautions and the low resistance of the protein depleted dog

It is obvious from the data of Tables 4 and 4-*a* that *cystine and tryptophane* are effective supplements for *protein digest X*, a digest which is not utilized by vein unless supplemented

Of some interest is a clear demonstration of the retention of the added

cystine (or cysteine) sulfur The urinary total sulfur during periods 5, 6, and 7 was 125 mg , 136 mg , and 129 mg , respectively, a satisfactory base line When cystine sulfur of 1495 mg was added in period 8, the sulfur excretion rose only to 186 mg The added *cystine sulfur* was more than *95 per cent retained* Plasma protein production increased sharply One might point out that the quantity of plasma protein removed during period 8 must have contained only about 160 mg of cystine sulfur The urinary

TABLE 4

Protein Digest X by Mouth or by Vein

Promotes Rapid Plasma Protein Formation

Only When Supplemented with Cystine and Tryptophane

Dog 39 106

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein emoved Total for 7 days	Blood plasma Average concentration		R.B C hema-tocrit average	Plasma volume	Weight
				Total protein	A/G ratio			
		gm	*gm*	*per cent*		*per cent*	*cc*	*kg*
	Kennel	—	—	6 43	0 93	—	—	11 5
1	Fasting	0	17 6	5 89	0 87	49 2	474	10 2
2	Low protein	3	30 4	4 49	0 75	50 7	356	10 2
3	Liver basal	73	28 8	4 13	0 64	49 0	429	10 0
4	Casein basal	96	20 4	3 86	0 54	47 4	429	10 2
5	Casein basal	96	18 4	4 06	0 67	50 3	424	10 2
6	Low protein + *protein digest X**	91	12 1	3 70	0 60	49 9	327	10 0
7	Low protein + *protein digest X*	93	8 4	4 02	0 51	47 8	323	9 7
8	Low protein + protein digest X + cystine + tryptophane by mouth	92	22 1	4 21	0 64	47 4	449	10 2
9	Casein basal	100	25 2	4 33	0 64	48 8	373	10 1
10	Low protein + *protein digest X + cysteine + tryptophane*	94	23 8	4 15	0 50	47 8	435	10 0

* Items in italics given intravenously

sulfur of period 9 was 146 mg and rose during period 10 to 176 mg when cysteine sulfur, 1478 mg , was injected by vein Again, the added sulfur was more than 95 per cent retained The conservation of this sulfur is associated with a large production of plasma protein Sulfur retention has been demonstrated under many other circumstances

Experimental History —Dog 39-316 (Table 5) An adult, female beagle hound was fasted 2 days (period 1) The low protein diet fed the remaining 16 two-day periods consisted of cane sugar 146 gm , lard 34 gm , butter fat 14 gm , salt mixture 6 gm , bone ash 6 gm , cod liver oil 10 gm , dried yeast 1 gm , choline hydrochloride 0 2 gm , nicotinic acid 0 025 gm This diet was eaten completely through period 4

During periods 4 and 5, casein digest L by vein was given, 30 gm per period in 3 equal doses as an 8 or 9 per cent solution, also containing glucose 4 per cent and sodium chloride 0 9 per cent In periods 6 and 7 the digest was increased to 40 gm per period, given in 4 equal doses In periods 8 and 9, *l* tryptophane, 1 2 gm , was added to 40 gm digest per period In periods 10 and 11, *l*-cysteine hydrochloride, 2 0 gm per period,

TABLE 4-*a*

Nitrogen Balance

Nitrogen Retention Only When Protein Digest X Is Supplemented with Cystine and Tryptophane

Dog 39 106

Period 7 days	Diet	Nitrogen balance							
		Intake		Output					Intake minus output
		In diet	In excess R.B.C. injected	In plasma	In feces	In urine			
						Total	Urea + NH_3	Undetermined	
		gm	gm	gm	gm	gm	per cent	gm	gm
	Kennel	—	—	—	—	—	—	—	—
1	Fasting	0	0 1	2 9	†	19 3	—	—	−22 1
2	Low protein	0 8	−0 5	5 0	3 9	14 2	—	—	−22 8
3	Liver basal	12 1	−0 3	4 7	1 5	14 5	—	—	−8 9
4	Casein basal	15 6	2 8	3 4	2 1	14 7	—	—	−1 8
5	Casein basal	15 6	1 5	3 0	1 8	15 6	77 8	3 6	−3 3
6	Low protein + *protein digest X**	14 6	1 3	2 0	1 9	22 9	74 2	6 0	−10 9
7	Low protein + *protein digest* X	14 7	5 5	1 3	2 0	22 5	71 6	6 3	−5 6
8	Low protein + protein digest X + cystine + tryptophane by mouth	15 6	8 0	3 6	3 3	11 9	72 7	3 2	+4 8
9	Casein basal	16 3	7 2	4 2	2 2	14 7	82 8	2 5	+2 4
10	Low protein + *protein digest* Y + *cysteine* + *tryptophane*	16 0	7 4	3 9	2 3	14 2	74 7	3 6	+3 0
	Totals	121 3	33 0	34 0	21 0	164 5	—	—	−65 2

† Included in following period

* Items in italics given intravenously

was added to this combination Reaction to these injections was minimal, a little mucus was vomited on only 2 occasions In periods 5 through 9 the oral diet was consumed 95 per cent, but in periods 10 and 11 half of the diet had to be spoon fed each day

In periods 12 and 13, casein digest L, 20 gm , *l* tryptophane 0 6 gm , and *l*-cystine 0 8 gm were mixed daily into the low protein diet and fed Appetite improved and spoon feeding was unnecessary in period 13

In periods 14 and 15, protein digest X 500 cc. with added tryptophane, 1.2 gm , and cysteine hydrochloride 2 0 gm , was injected intravenously, in 4 equal doses in period 14 and in 8 equal doses in period 15 Vomiting of mucus occurred with several

of the injections, even when given at a rate of 2 to 3 cc per minute Spoon feeding achieved 100 per cent consumptions of the oral diet Period 16 was of only 1 day's duration but the data in the table are adjusted to a basis of 2 days

During period 17, *heparinized dog plasma* was given by vein in 2 injections daily, totaling 5 08 gm nitrogen The oral diet had to be entirely spoon fed

TABLE 5

Casein Digest L Utilized by Normal Dogs

Period 2 days	Diet	Total N Intake	Dog 39-316—Urine				Dog 39-307—Urine			
			Total N	Urea + NH_3 N		Undetermined N	Total N	Urea + NH_3 N		Undetermined N
		gm	*gm*	*gm*	*per cent*	*gm*	*gm*	*gm*	*per cent*	*gm*
1	Fasting	0	9 49	8 12	85 6	1 37	6 80	5 91	86 8	0 89
2	Low protein	0 16	4 79	3 44	71 8	1 35	4 82	3 67	76 1	1 15
3	Low protein	0 16	3 37	2 50	74 1	0 87	4 63	3 50	75 6	1 13
4	Low protein + *digest L**	3 90	5 85	3 63	62 2	2 22	6 62	4 47	67 7	2 25
5	Low protein + *digest L*	3 90	5 52	3 70	67 0	1 82	8 50	6 42	72 9	2 08
6	*Low protein + digest L*	*5 16*	*5 87*	*3 53*	*60 2*	*2 34*	*8 97*	*5 92*	*66 0*	*3 05*
7	Low protein + *digest L*	5 16	6 30	3 98	63 1	2 32	7 28	4 82	66 2	2 36
8	Low protein + *digest L* +	5 33	6 04	3 86	63 9	2 18	7 07	4 41	62 4	2 66
9	*tryptophane*	5 33	6 60	4 14	62 7	2 46	7 01	4 35	62 0	2 66
10	Low protein + *digest L* +	5 51	7 00	4 54	64 9	2 46	6 14	3 71	60 5	2 43
11	*cysteine* + *tryptophane*	5 51	5 92	3 54	59 8	2 38	6 27	3 81	60 8	2 46
12	Low protein + digest L +	5 51	3 40	2 24	65 9	1 16	3 40	2 27	66 7	1 13
13	cystine + tryptophane	5 51	3 96	2 76	69 7	1 20	3 58	2 42	67 5	1 16
14	Low protein + *digest X* +	5 51	6 60	4 22	64 0	2 38	6 56	4 17	63 7	2 39
15	*cysteine* + *tryptophane*	5 51	5 00	3 03	60 5	1 97	6 00	3 84	64 0	2 16
16	Low protein	0 16	2 48	1 54	61 8	0 96	3 16	2 04	64 6	1 12
17	Low protein + *plasma protein*	5 22	2 45	1 54	62 9	0 92	3 46	2 50	72 2	0 96

* Items in italics given intravenously

Weight varied little after the start of the experiment 9 8 kg in period 5, 9 6 kg in period 13, 9 8 kg in period 17 This dog was the subject of later experiments, Tables 2 and 2-*a*

Experimental History —Dog 39-307 (Table 5) An adult female, mongrel, long haired terrier was fed the same diet and given the digest and amino acid mixtures in the same amounts and in the same fashion on the same days as dog 39-316 Since the oral diet was eaten only 10 to 30 per cent during periods 2 to 4, it had to be spoon fed for complete

consumption during the remainder of the experiment. In the course of the first casein digest L injection, there was some restlessness, weakness of pulse, and vomiting Some mucus was vomited following the injection of protein digest X, period 14 Small amounts of the low protein diet were vomited following spoon feedings on 3 occasions, periods 14 16 and 17 The amount of dog plasma given in period 17 was practically the same as that given dog 39 316 (5 04 gm nitrogen) Weight varied little after the start of the experiment 11.3 kg in period 6 and 11 1 in period 17

In Table 5, both digests (casein L and protein X) were tested in 2 normal dogs not subjected to plasmapheresis Casein digest L was well utilized by mouth, periods 12 and 13 It was utilized by vein but not as well as is demonstrated above in the plasma depleted dogs For example, in dog 39–316, for 16 days during digest injection (periods 4 through 11) the nitrogen intake was 39 8 gm , and the nitrogen output (assuming fecal nitrogen of 0 5 gm per period) was 53 1 gm This negative balance of 13 3 gm or 5 8 gm per 7-day period may be compared with negative balances obtained in this dog when no digest was being given For period 2, Table 2 *a*, the negative balance was 10 0 gm per 7 day period It would appear that some of the nitrogen of the intravenous digest had been retained Cysteine and tryptophane obviously do not improve this retention The protein digest X is similarly ineffective (periods 14 and 15) The slower rate of injection in period 15 (see Experimental History) did not improve nitrogen retention This difference in the digest nitrogen retention of plasma depleted dogs as compared with that of non depleted dogs is being investigated

Intravenous plasma protein again proves its nutritional value (period 17) Its nitrogen is completely retained in both dogs The response to both digests is the same in dog 39-307 as in dog 39-316, except that dog 39-307 may not have been entirely normal during the early part of the experiment (see Experimental History, dog 39-307) If some intoxication existed, it seems proper to refer it to the factors responsible for the poor food consumption prior to digest injections and to the higher concentration of digest used, unfiltered and unsterile, not the water clear solution of the 5 per cent concentration

DISCUSSION

Perhaps it is not for us to debate clinical problems but we may mildly suggest that the experiments designed to study plasma protein production do have a bearing on the many sided problem of *shock* as well as clinical *hypoproteinemia* If the body can be aided in producing new plasma proteins this procedure may be as valuable as the administration of plasma by vein Plasma protein by vein is most effective to correct emergency hypo

proteinemia and to furnish protein to depleted or injured body cells But casein digests by vein or subcutaneously are as effective as protein by mouth in building new plasma protein and when protein cannot be eaten the digest can wholly replace food protein for many weeks It may be that *casein digests* can be used with profit to *supplement plasma injections* or even to replace the intravenous plasma when the acute emergency is passed The advantages of the casein digest for clinical use scarcely need mention It is non-toxic and can be stored in concentrated form It is inexpensive and obtainable in unlimited amounts and can be readily sterilized

SUMMARY

When blood plasma proteins are depleted by bleeding with return of the washed red cells (plasmapheresis) it is possible to bring dogs to a steady state of hypoproteinemia and a uniform plasma protein production on a basal diet limited in protein Such dogs are clinically normal but have a lowered resistance to infection and certain intoxications

Casein digests given by vein or subcutaneously to such plasma depleted dogs are effective in promoting abundant new plasma protein production Casein digest L by vein is equivalent to whole liver of like protein equivalence by mouth The ratio of new plasma protein production to protein intake is 20 to 25 per cent in both instances

Casein digest L by vein gives the same response in plasma protein output as the same digest by mouth Protein digest X by vein requires addition of tryptophane and cysteine to be effective in plasma protein production The added cysteine sulfur is more than 95 per cent retained by the dog

The speed of digest injection has no effect on its utilization, within the range tested

Casein digest L given by vein to non-depleted dogs is less well utilized than in dogs depleted of plasma protein

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A SIMPLIFIED PERFUSION APPARATUS FOR THE MAINTENANCE OF LIVING ORGANS IN VITRO

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PLATE 34

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In this paper an apparatus will be described for the perfusion of organs It fulfills the necessary requirements for this type of experiment and can be so easily constructed and regulated as to function with a maximum of ease

The apparatus has the original characteristics of producing constant pulsatile circulation without resort to valves either in its circuit or in its pump It has, in addition, a double system of oxygenation

Description of the Apparatus

The Perfusion System—The perfusion system (Text fig 1 semi schematic, Text fig 2, as set up) consists of a principal chamber (*A*) (a broad dish supported on a tripod) to house the organ—into which is inserted a glass cannula with a fine opening (*4*)—and two small glass chambers placed on a lower level, one an intermediate chamber (*B*) and the other a pressure chamber (*C*) communicating with the principal one The first of these chambers is joined directly to *A* by means of a narrow tube (*1*) through the floor (*3*), and the second by means of a tube (*2*) coming from the middle of the chamber and connecting with the cannula (*4*) The two small chambers communicate *via* the pump (*D*)

The organ chamber (*A*) possesses a tube (*5*) which connects with the oxygenation system A glass plate (*E*), the edges of which are covered with a thin film of oil, hermetically seals the container (*A*) and is held in position by means of a steel wire clamp (*e,e'*)

The intermediate chamber (*B*), as well as its connections (*1*) with the organ chamber and the pump to be described further on, has joined to it a funnel (*F*) by a tube (*6*) which opens into the upper part of the chamber A stopcock (*7*) enables the communication between the funnel (*F*) and the chamber (*B*) to be opened and closed

To absorb the CO_2 coming away from the perfused organ, an open ended glass tube containing calcium hydrate (*8*) projects into chamber *B* The lower end of the tube is

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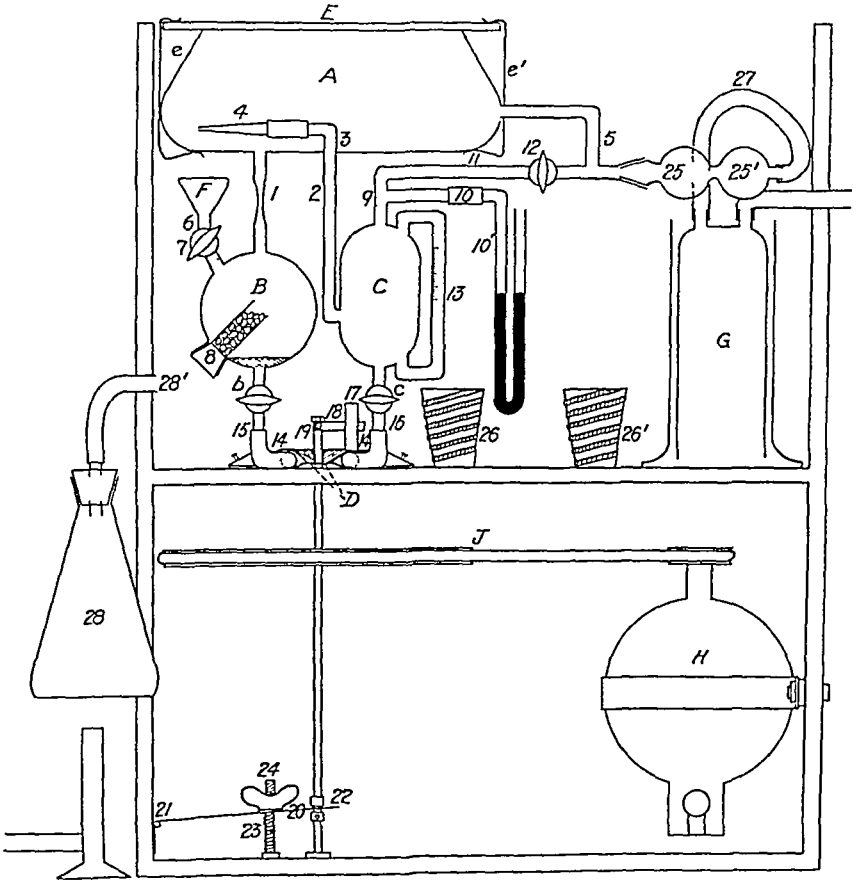
I wish to thank Dr J H Means for the facilities he has given me in his laboratory and for his supervision of my work I am also indebted to Dr S Hertz Mr Raul Adelman and Dr C K Drinker for their useful advice in the construction of the apparatus

closed with a rubber stopper, and the same rubber stopper fits into and seals the opening through which the glass tube has been introduced into the chamber

Filtration of the perfusate, as it leaves chamber *B*, is accomplished by means of a layer of sand supported on a layer of cotton gauze to prevent it from falling into the tube connecting chamber *B* with the pump

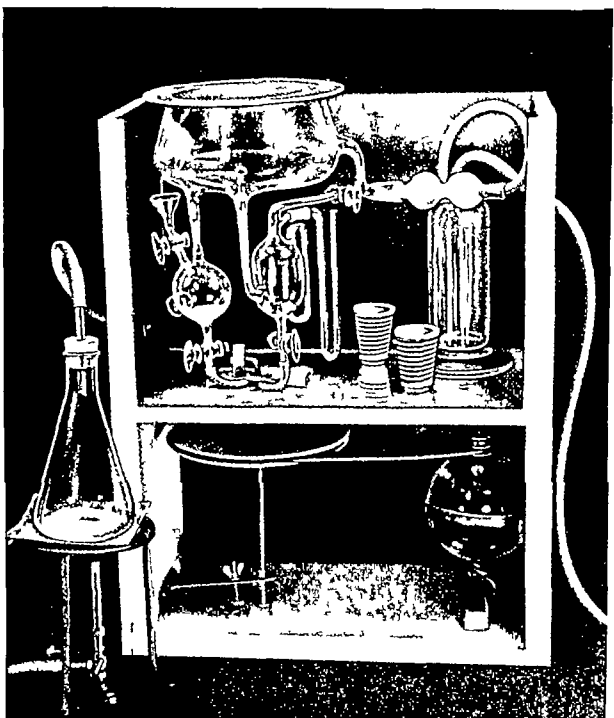


TEXT-FIG 1 Schema of whole apparatus

The pressure chamber (*C*), of cylindrical form, has in addition to the tubes that connect it with the pump (*D*) and the organ chamber (*A*), a bifurcated tube (*9*) in its upper part, one branch of which (*10*) is connected with a manometer (*10'*) and the other (*11*) with another filter (*25, 25'*) A stopcock (*12*) permits the establishment or interruption of these communications A tube (*13*), almost capillary in type, leaves the top of the chamber (*C*) and enters it again from the bottom This tube is used to measure the quantity of oxygen consumed by the organ A graduated scale in its wall facilitates measurement of the pressure Its function will be explained later

The essential part of the apparatus is the pump (*D*) which is a piece of ordinary rubber

tubing (*14*) placed on the floor of the box which contains the apparatus It is horse shoe shaped (*i e* an incomplete circle) and the ends (*15 16*) are connected with the intermediate (*B*) and pressure (*C*) chambers respectively



TEXT FIG 2 The perfusion apparatus in actual operation

A solid brass wheel (*17*) rotates on the horseshoe shaped rubber tubing compressing the rubber during its revolutions The fluid contained within the tubing is forced ahead of it into the pressure chamber (*C*) and at the same time the fluid in the intermediate chamber (*B*) is subjected to the resulting negative pressure thus providing for a return of fluid like the venous return in an animal s circulation

The wheel (*17*) is mounted on a short horizontal axle (*18*) supported by a long vertical axle (*19*) which is connected to the motor (*H*) by means of a belt (*J*) The horizontal axle (*18*) is fixed to the vertical (*19*) by means of a screw which facilitates the removal of the wheel when it is necessary to take out the pump The long vertical axle (*19*) can be moved up and down, thus changing the pressure of the wheel on the rubber tubing, which in turn allows wide variation of the pressure of the fluid A lever (*20*), fixed in place at one end (*21*) and attached at the other end (*22*) to the axle (*19*) tends to exert an upward pressure on the axle through the action of a spring (*23*) A nut (*24*) fixes the lever, and hence the axle (*19*), in the desired position for the production of any given pressure The motor driving the wheel is regulated by a rheostat The maximum speed is 200 revolutions per minute The two stopcocks (*b* and *c*) are used when it is necessary to change the pump By closing these it is possible to remove the pump and replace it by a new one

System of Oxygenation —This consists of a spirometer (*G*) placed inside the box which contains the remainder of the apparatus and connected with the latter by means of a rubber tubing (*27*) The gas mixture flows through the filters (*25* and *25'*), through the branches (*5* and *11*) into the organ chamber (*A*) and pressure chamber (*C*) The gas mixture is under slight pressure from the weight of the bell of the spirometer

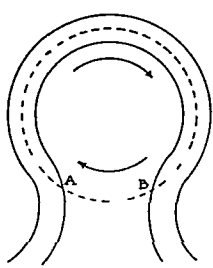
Temperature —A nearly constant temperature is maintained in the box containing the apparatus since it has double walls of monel metal with insulation of asbestos between The side walls and floor are fixed in relation to the other panels, which are movable, and they slide into the permanent part of the box by means of grooves The axle (*19*) of the pump passes through the center of the floor of the box Describing a rectangle around this center are four nails to fix the pump The thermometer is introduced through an opening in the top wall An opening (*28'*) in the side panel is provided to allow a steam-conducting tube to enter the box for use during sterilization of the apparatus *in situ* The front panel contains an opening into which either a piece of double glass or an insulated panel may be inserted the former to view the apparatus in motion and the latter to close it for sterilization Two electrical heating elements (*26* and *26'*) are connected to a rheostat and provide a temperature range from the running temperature to one of 150°C for sterilizing purposes

Function —The fluid is driven through the organ by the impulse given to it by the pump The fluid is forced from the pump (*D*) to the pressure chamber (*C*) where it is oxygenated under pressure It leaves the chamber (*C*) through the tube (*2*), goes into the organ chamber (1), and through the cannula (*4*) (previously attached to the end of the tube (*5*)) perfuses the organ During the course of the perfusion from the pump to the organ, the pressure of the fluid may be so regulated that it possesses systolic and diastolic variations approximating those which obtain in the arteries of the organ The fluid leaves the organ freely under no pressure In the organ chamber (*A*) it is again in contact with the gas mixture The floor of the chamber is somewhat inclined, so the fluid flows into the tube (*1*) through which it is aspirated toward the intermediate chamber (*B*) by the negative pressure created by the pump (*D*) (The inclination of the floor of the chamber is not shown in Text-fig 1, because the schema is a cross section at right angles to the plane of inclination) In the intermediate chamber (*B*) it flows down the walls in a thin sheet and accumulates at the bottom before returning to the pump The reduced pressure existing in this chamber facilitates the passage of the CO_2 contained in the fluid into the atmosphere of the chamber where it is absorbed by the calcium hydrate contained in the tube (*8*)

Pulsations—Text fig 3 shows the horseshoe shaped rubber tubing (double line) and the course (broken line) on which the brass wheel travels The wheel compresses the tubing as it passes and pushes the fluid ahead of it. Thus a certain pressure is imparted to the fluid, which, however, is partially lost when the segment *B A* is reached In this fashion a pulsatile flow is achieved The number of pulsations per minute depends on the rapidity of revolution of the wheel which can be regulated by the rheostat controlling the motor With this system, it is possible to obtain pulsations without any valves

Systolic or Maximum Pressure—The systolic pressure is regulated (see Text fig 1) by means of a screw (*24*) on the lever (*20*) which raises or lowers the vertical axle (*19*) so that the wheel (*17*) produces the desired pressure on the rubber tubing and therefore determines the efficiency of the action of the pump If the axle (*19*) is elevated the pressure will drop The reverse is true if the axle is lowered

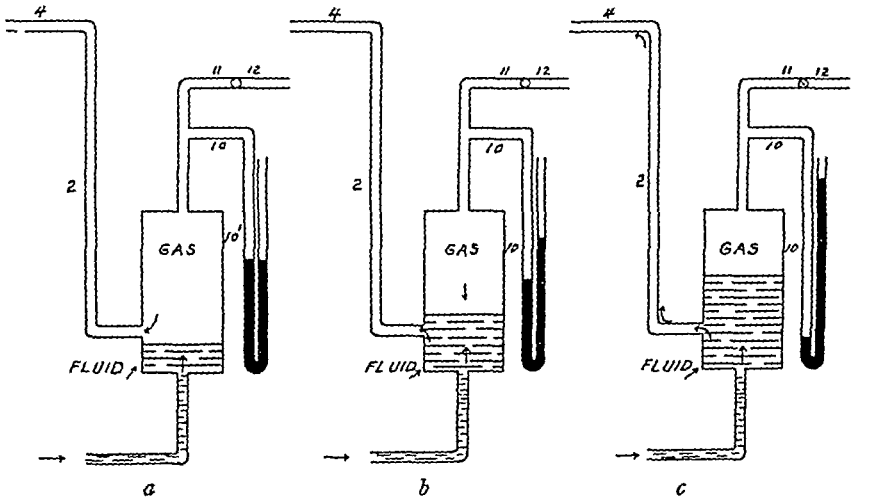
Diastolic Pressure—*The diastolic pressure is produced by the drop of pressure that* takes place when the wheel runs its negative course from *A* to *B*, Text fig 3 Its regulation takes place in the pressure chamber (*C*) If the communication of the pressure



TEXT FIG 3 Diagram indicating the principle of the pump

chamber with the gas mixture tank is interrupted by use of the cock (*12*), the chamber will have only one communication with the exterior (Text fig 4, *a*) and that is *via* the tube (*2*) which goes to the organ chamber When the pump begins to force the fluid into the organ chamber (*C*) the level rises until the tube s opening is covered (Text fig 4 *b*) At this point the gas mixture begins to be compressed because the amount of fluid forced in by the pump is more than the amount that goes out *via* the narrow cannula (*4*) The pressure rises the fluid goes up into the tube (*2*) and perfuses the organ When the maximum desired pressure is reached (Text fig 4 *c*) (see regulation of maximum systolic pressure) two different media are found in the pressure chamber one incompressible (fluid), that will transmit without variation all the differences produced by the pump, and the other, compressible (gas), which will tend to absorb the variations and to transform the pulsating circulation into a continuous one contracting with any rise and expanding with any diminution of the volume of liquid Consequently the efficiency of this "shock absorber depends on its volume If the amount of gas is reduced the efficiency will diminish and the difference in pressure will not be absorbed The reverse will take place if the volume of the gas mixture rises Then, to increase the differential pressure the cock (*12*) is opened, thus allowing the gas to go from the chamber (*C*) to the tank (*G*) (Text fig 1)

To decrease the differential pressure the following procedure is carried out The pump is stopped, the wheel is put in the negative path, and the cock (*12*) is opened The fluid will then move only by a difference of level from the pressure chamber to the intermediate chamber, and at the same time the gas will go from the tank (*G*) into the pressure chamber (*C*) When the fluid reaches the desired level the cock (*12*) is turned off and the pump is put to work again During all these operations care must be taken that the level of the fluid does not fall below the upper edge of the opening of the tube (*2*), because if the gas penetrates into the tube it will produce an embolism in the organ With this system we have been able to obtain differences in pressure between the minimum of 2 mm and the maximum of 80 mm of mercury



TEXT-FIG 4, *a*, *b*, and *c* Diagram indicating regulation of differential pressure in chamber (*C*)

Oxygenation —Mixture of gases takes place in the tank (*G*), and from there it passes through the filters (*25* and *25'*) to the organ chamber (*A*) and the pressure chamber (*C*) In the organ chamber (*A*) the gas pressure will be the same as in the tank, in the pressure chamber (*B*) it will be in equilibrium with the perfusion fluid The efficiency of the oxygenation in this chamber is due in part to the existing pressure and in part to the time that the fluid, in continuous movement, remains in contact with the gas mixture

Absorption of Carbon Dioxide —The carbon dioxide is taken up by the calcium hydroxide in the tube (*8*) of the intermediate chamber (*B*) It passes from the fluid, in which it is dissolved, to the air of the chamber because of the negative pressure produced by the aspirations of the pump In order to maintain this negative pressure, the intermediate chamber (*B*) is necessarily separated from the organ chamber (*A*) because the latter contains gas at 2 or 3 mm pressure (the same as in the tank) For this reason the tube (*1*) is almost capillary The fluid, continuously streaming through this constriction, separates the atmosphere of the two chambers and permits the maintenance of a negative pressure in the intermediate chamber at the same time that a positive pressure exists in the organ chamber

Temperature—Heat is produced by the electrical elements (*26* and *26'*) It is regulated by a rheostat, maintained by the characteristics of the box and registered by a thermometer

Evaporation—Evaporation must necessarily be avoided to prevent change in concentration of the fluid For this reason the box must have a constant temperature throughout, which will prevent condensation of moisture on the glass walls that would occur if one portion were slightly colder than the rest

Measurement of Oxygen Consumption—The oxygen consumed can be measured in the following way The oxygen in the pressure chamber (*C*) during function of the apparatus is constantly being utilized by the organ and transformed into carbon dioxide During its consumption the volume of the oxygen in the pressure chamber (*C*) maintained at the same pressure and temperature, will diminish Hence the level of the fluid will rise Reading the difference between the levels in the tube (*13*) in a given time it is possible to calculate the volume of the oxygen consumed in the chamber and to relate it to the normal pressure and temperature of the atmosphere

Cleaning and Sterilizing—By disconnecting the rubber tubing (*27*) which joins the apparatus to the tank (*G*) and by removing the short axle (*18*) from the long axle (*19*) *the entire apparatus can be separated from the box containing it and can be cleaned* under running water (after the cover (*E*) of the organ chamber (*A*) has been removed) One of the ends (*15* or *16*) of the rubber tubing of the pump must be disconnected to permit the water to pass from the organ chamber through the whole system and out through the disconnected end. After 2 hours under running water the apparatus should be washed once more, this time in distilled water and the pump connected again The apparatus is then put in the box and the box closed The temperature is raised to 150°C by means of the electrical heating elements At the same time the atmosphere of the box and of the apparatus is saturated by means of a steam producer (*28*) for 4 hours (No rise in pressure is produced in the box because it is not completely air tight) After this procedure one can be sure that the apparatus is sterilized One should remember that the atmosphere must be completely saturated with steam not only to insure sterilization but to prevent deterioration of the rubber

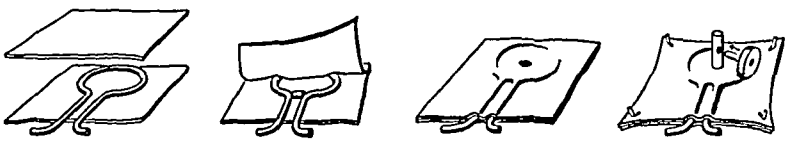
To dry the apparatus the production of steam and the heat from the elements is stopped and the pump put in motion Because the pump, on producing a movement of air expels the steam the top panel of the box should be slightly opened and the residual heat will dry the apparatus During all the processes of sterilization and drying the top (*E*) of the organ chamber (*A*) should be slightly raised to allow free passage of steam When the apparatus is dry the cover of the organ chamber should be closed and the box sealed It should remain so until it is to be used It is important to wash and dry the apparatus as indicated in order to remove all small particles that might cause embolism of the small arteries or capillaries of the organ

How to Operate the Apparatus

The technique of operation on the animal (with slight modification), the perfusion fluid, and the gas mixture used are the same as those advised by Carrel in his experiments To make the apparatus function properly, these steps should be followed

the artery, a ring of rubber tubing is placed in the middle of the cannula and the two ends of the silk used to tie the vessel to the cannula are fixed in place by inserting them into two cuts made in the ring of rubber (Text-figs 5, 6, and 7) The cannula (*4*) is inserted into tube (*3*) by means of another rubber ring

Construction of the Pump —The rubber tubing which constitutes part of the pump requires for perfect function a fixed position on the floor of the box so that the wheel can turn and operate efficiently on it The rubber tubing is glued between two rubber patches of rectangular shape For this purpose common rubber tire-repair patches are used Two rectangular pieces are cut out and the rubber tubing, well covered with rubber cement, is attached in a horseshoe shape of proper diameter (Text-fig 8) to one of the patches The second patch is cemented on top (Text-fig 9) and the rubber tubing which is an integral part of the pump is thus fastened in place The whole is placed under a weight until dry Then a hole is made in the center of the horseshoe through which the long vertical axle passes (Text-figs 10 and 11) and the edges of the hole are sewed together The rubber rectangles are fixed by their corners to the floor of the box by means of nails (Text-fig 11) The ends of the rubber tubing are connected with the



8 9 10 11

TEXT-FIGS 8, 9, 10, and 11 Schema showing the construction of the pump

corresponding chambers As previously stated, a pump of this sort will last for 4 days of continuous operation, after which it has to be replaced

Two details should be remembered First, the rubber tubing must be thoroughly washed and boiled in alkaline water, and last of all, a small piece of rubber tubing should be attached in the negative transverse to avoid jumping of the wheel (Text-fig 9)

FIGS 2, 4, and 6 show sections of two lobes of a thyroid and a suprarenal which were perfused for 3 days at the same time in the apparatus There were no perceptible histological changes on comparison with control specimens of the organs fixed when freshly taken (FIGS 1, 3, and 5)

SUMMARY

A simplified perfusion apparatus for the maintenance of living organs *in vitro* is described

APPENDIX

Below we give dimensions of some parts of the apparatus, dimensions of other parts which need not have such definite sizes have been omitted

Organ chamber	20	cm diameter	6	cm	height
Intermediate chamber	6	" "	7	"	"
Pressure chamber	6		7	"	"
Tubes (*1*) and (*2*)	5	" "			
Tubes (*b*) and (*c*)	3				
Circle of rubber tubing	2½	" "			
Wheel	3	'	1½	"	width
Glass tube inside diameter	4	mm			
Cannula length	7	cm			

Rubber tubing used for pump, inside diameter, 3 mm , width of wall, 1 mm

EXPLANATION OF PLATE 34

The sections were stained with hematoxylin and eosin × 125.

FIG 1 Thyroid control

FIG 2 Thyroid perfused

FIG 3 Thyroid control

FIG 4 Thyroid perfused

FIG 5 Suprarenal control

FIG 6 Suprarenal perfused

(Galli Mainini: Perfusion apparatus for living organs)

NATURE OF NON-PARALYTIC AND TRANSITORY PARALYTIC POLIOMYELITIS IN RHESUS MONKEYS INOCULATED WITH HUMAN VIRUS*

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PLATES 35 TO 38

(Received for publication, March 7, 1941)

The existence of non paralytic or abortive poliomyelitis, although suspected, has not previously been established in monkeys either by histologic examination or by isolation of the virus on passage In human beings the term "abortive poliomyelitis" has been applied to cases of minor illness, exhibiting usually headache, fever, vomiting, and sore throat, but not signs of involvement of the central nervous system, occurring in families or groups in which paralytic poliomyelitis was present The isolation of poliomyelitis virus from the oral washings of two such cases by Paul and Trask (1) and the more recent demonstrations of virus in the stools of such patients (2–5) established on a firm basis what had long been suspected on clinical and epidemiological grounds The term "non paralytic poliomyelitis" has been used in human beings to designate cases which, in addition to the symptoms listed above, exhibit stiffness of the neck and back, pleocytosis, tremor, and other signs and symptoms of preparalytic poliomyelitis but never develop paralysis Again the demonstration of virus in the stools of such patients in recent years (2–5) has provided proof of the true poliomyelitic nature of the non paralytic disease Although it is commonly stated that the central nervous system is affected in the non paralytic but not in the abortive form of human poliomyelitis, little or nothing is known of the actual nature or extent of involvement of the central nervous system in either form of the disease

Non paralytic or abortive forms of experimental poliomyelitis have been recorded but rarely and then on equivocal grounds Harmon, Shaughnessy, and Gordon (6), using fever, pleocytosis of more than 150 cells per c. mm , increase in cerebrospinal fluid globulin, neutrophilic leucocytosis in the peripheral blood, and the usual preparalytic signs (excitability or apathy, tremors weakness, etc) without development of paralysis

* Aided by a grant from the National Foundation for Infantile Paralysis Inc

as criteria, observed what they considered to be the abortive form in 10 out of 555 inoculations in 350 monkeys They used monkey passage virus and the 10 animals were for the most part inoculated intracerebrally with serum-virus mixtures No histological examination of the nervous system is recorded, but 5 of 9 monkeys tested for immunity succumbed with typical paralysis on subsequent inoculation of virus A recent report by Kling (7) stated that *rhesus* and *cynomolgus* monkeys inoculated with human material (or drinking water) exhibited "mild paralytic" or "abortive" forms of poliomyelitis in which *no neuronal lesions* were present but only cellular infiltration of the vessels, usually those of the meninges or choroid plexus This report did not give adequate evidence that those lesions were caused by poliomyelitis virus or any other virus, and will be discussed later on in the light of our own findings

In the beginning of a study on the distribution and elimination of virus in human poliomyelitis it was observed that of 2 monkeys inoculated with the same specimen of stool by different methods, 1 developed typical paralytic poliomyelitis while the other exhibited only some transitory fever and questionable tremors When the latter monkey was sacrificed 30 days after inoculation, typical poliomyelitis lesions, no longer acute in character, were discovered throughout the neuraxis In subsequent studies 287 inoculated monkeys which failed to develop paralysis, were sacrificed at the end of 30 to 37 days and their nervous system was submitted to histological examination The purpose of this communication is to present evidence that non-paralytic poliomyelitis occurs in monkeys inoculated with human material and to point out (*a*) that it is almost invariably associated with the destruction of an appreciable number of nerve cells in the spinal cord, (*b*) that its failure to progress to distinct paralysis depends upon an equilibrium between the virus and the host, in which the virus is not always rapidly destroyed since it can occasionally be passaged to other monkeys, and (*c*) that transitory paralysis depends upon a similar equilibrium, in which, however, it is not clear whether a certain number of cells attacked by the virus recover or whether the remaining nerve cells become sufficient for apparently normal function

Methods

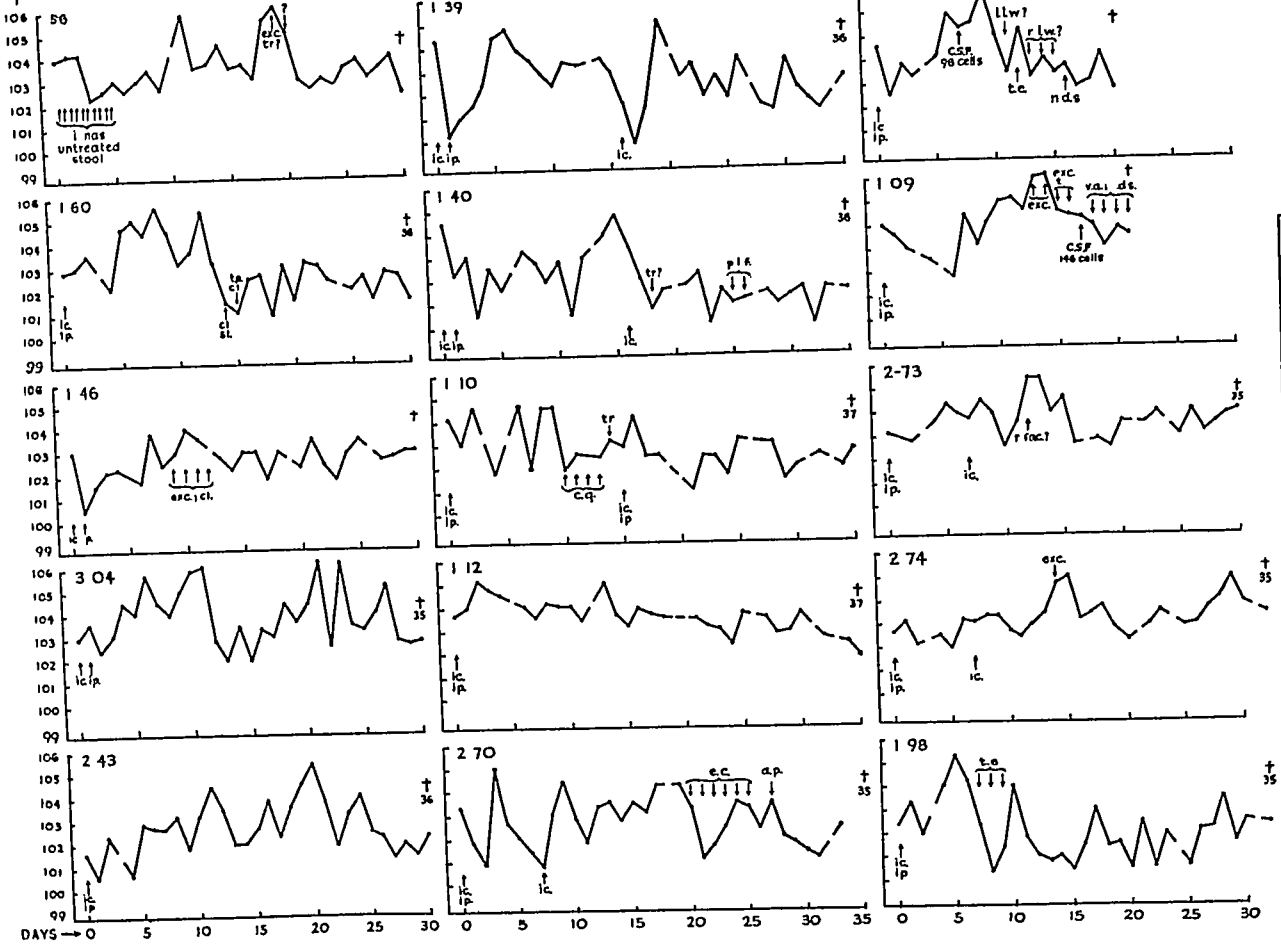
All inoculated monkeys were observed daily, or oftener if they exhibited suspicious signs of any kind Their rectal temperature was taken daily for 30 to 35 days and they were exercised in sufficiently large enclosures to permit observation of abnormal running or climbing Special care was taken to test for deltoid and facial paralysis When the monkeys were sacrificed, usually 35 days after inoculation, their viscera were examined for gross pathological changes, especially for those of a tuberculous nature, and it is pertinent to state that only 2 of 400 monkeys exhibited gross evidence of tuberculosis The entire brain and cord were examined macroscopically and six levels of the spinal cord and one of the medulla in the region of the olivary bodies were studied micro-

scopically as routine Three lumbar one thoracic, and two cervical levels of the spinal cord were usually obtained, care being taken to include the roots, in which the most striking evidence of previous neuronal destruction is often to be found In addition one or two regions of the diencephalon just caudal to the optic chiasma were also studied in many instances and the olfactory bulbs and frequently the anterior perforated substance were included whenever a monkey had received nasal instillations Tissues for histological study were fixed in the Zenker acetic mixture and as a rule stained with eosin and methylene blue Part of the spinal cord and medulla of each monkey were stored in 50 per cent buffered glycerol at approximately 5°C , to permit passage to other monkeys whenever it was deemed necessary or desirable

RESULTS

The diagnosis of non paralytic poliomyelitis was made in 16 instances Among 157 monkeys, which failed to develop paralysis following inoculation with various human tissues or with first passage virus, there were 14 with non paralytic poliomyelitis, 11 of these were inoculated with human tissues and 3 with the nervous tissue of monkeys paralyzed by human virus Only 1 case of non paralytic poliomyelitis was found among 23 presumably negative monkeys which had received nasal instillations of untreated stools (8) or intraabdominal injections of etherized stool suspensions (5), or both The remaining case of non paralytic poliomyelitis was found in 1 of 3 monkeys which apparently failed to react when passage of virus obtained from human stools was attempted in them Among 54 non reacting monkeys, inoculated with nasal secretions, saliva, or urine from poliomyelitis patients, and 44 "negative" monkeys, which received various tissues of other monkeys inoculated with M V virus, none exhibited the lesions which warranted the diagnosis of non paralytic poliomyelitis

Clinical Course of Monkeys with Non Paralytic Poliomyelitis —The temperatures and clinical observations recorded in Chart 1 reveal a variety of responses which are so atypical that in the majority of instances it would not have been possible to predict what the histological study revealed The temperatures were sometimes distinctly elevated for a number of days but usually were quite irregular an occasional monkey was excited, tremulous clumsy, or questionably weak for a few days while many others appeared entirely normal. In the beginning many cell counts were done on the cerebrospinal fluid obtained by cisternal puncture but this procedure was abandoned later on because it could not be relied upon for diagnosis We have even encountered as many as 500 mononuclear white cells per c mm of cerebrospinal fluid in a monkey which on histological examination exhibited no poliomyelitis lesions, and whose nervous tissue upon inoculation into mice did not yield the virus of lymphocytic choriomeningitis or any other infectious agent A comparison between the temperatures and clinical course of a number of monkeys which were histologically negative (Chart 2) with those of monkeys in which the lesions of poliomyelitis were present, reveals that on the basis of clinical observations alone it is usually not possible to make the diagnosis of non paralytic



a.	arm
ap	apathetic
cl	clumsy
cq	climbs queerly
c.s.f	cerebrospinal fluid white cells per cu
e.c.	- easily caught
exc.	excited
ic.	- intracerebral inocu
i nas	nasal instillation
ip	intraabdominal ino
l l	left leg
n.d.s.	no definite sign
p	partial
par	- paralysis
p l f	- protects left foo
r fac	right facial para
r l	right leg
s l	- slow
t.e	tires easily
tr	- tremor
va	very active
w	weakness
† 36	sacrificed, 36th

CHART 1 Temperature charts of *rhesus* monkeys with non-paralytic poliomyelitis

poliomyelitis in monkeys The irregular temperature curves appeared to be correlated more with activity and climatic conditions than with anything else we could discover, since these monkeys were singularly free from tuberculosis and other organic diseases with the exception of the common benign esophagostomum infections of the gut and mild parasitic infestations of the lung When a monkey accidentally sustained a fracture

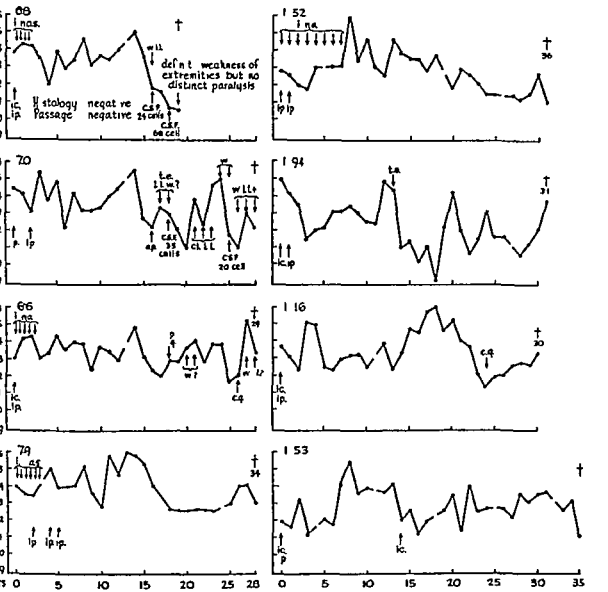


CHART 2 Monkeys with no histological evidence of poliomyelitis, but with temperatures or clinical course resembling those of non paralytic group

of a lower extremity or was continually kept in its cage even when its temperature was taken, remarkably flat temperature curves could be obtained

Criteria for Pathological Diagnosis of Non Paralytic Poliomyelitis and Results of Passage—Monkey 5-6 (Chart 1) was the first one to be examined and the changes in its nervous system indicated the extent and character of the lesions that might be found in a non paralytic animal The lesions were no longer acute in character in the sense that no acidophilic necrosis of nerve cells was present, and that there were practically no polymorphonuclear leucocytes in the foci of neuronophagia or among the cells infiltrating the interstitial tissue However this particular case was still sufficiently early to show more extensive infiltration and neuronal changes than were found in monkeys which had

apparently lived for a longer interval after the acute phase Monkey 5-6 which had been inoculated only by the nasal route and which was sacrificed probably about 10 days after the acute phase (judging from the number of days with entirely normal temperature and physical condition) exhibited the following changes (1) one of the olfactory bulbs was normal while the other presented marked infiltration with mononuclear cells especially in the glomerular layer, (2) there was a unilateral lesion in the anterior perforated substance with focal neuronophagia (*i e* glial nodules) and perivascular cuffing, (3) similar foci of neuronophagia and perivascular cuffing were also present in the hypothalamus, thalamus, mesencephalon (red nucleus), medulla, and in one anterior horn of the cervical cord, (4) the most extensive lesions, however, were present in the anterior and lateral horns of the sections of lumbar cord (Figs 1 and 2) which exhibited marked perivascular cuffing, disappearance of an appreciable number of nerve cells in the anterior and lateral horns with considerable focal and diffuse cellular infiltration in the areas of outfall of cells, and a large number of degenerated anterior horn cells with marginated Nissl substance and eccentric nuclei The nerve roots showed only minimal signs of degeneration at this stage

In another instance in which the monkey (7-6, Chart 1) was sacrificed relatively early, one could find almost complete destruction of most of one anterior horn of the cervical cord (Fig 3) with extensive interstitial infiltration and marked perivascular cuffing, an area of necrosis and neuronophagia was present in one lateral horn of the thoracic section and focal neuronophagia and perivascular cuffing in the medulla, in the lumbar sections the nerve cells appeared to be well preserved and only marked perivascular cuffing of vessels in the gray matter was present In the few monkeys sacrificed at this early stage the reaction of degeneration in the nerve roots was still not far enough advanced to be conspicuous In practically all the monkeys sacrificed beyond 30 days, however, wherever there was any suggestion of outfall of cells one could find reliable confirmatory evidence in the degenerative reaction which was present only in the corresponding nerve roots Thus, in Fig 4, one can see a section of the cervical cord of monkey 1-10 in which the nerve cells of the lateral half of one anterior horn have been destroyed with degeneration in the anterior roots of the same side, but not in those of the opposite side where all the nerve cells in the anterior horn may be seen to be intact It is interesting to note that in the same monkey no lesions were found in another level of the cervical cord, thoracic cord, or medulla, while in three levels of the lumbar cord only two small foci of interstitial infiltration and some perivascular cuffing were present The changes shown in Figs 3 and 4 are especially conspicuous even at low magnification because in addition to the disappearance of nerve cells there has been destruction or distortion of the ground substance by the inflammatory reaction In Fig 5 is shown a cord (monkey 1-46) in which the gray ground substance of the affected anterior horn appears well preserved and there is relatively little interstitial infiltration, but that a large number of the anterior horn cells is missing is obvious not only by comparison with the anterior horn of the opposite side but also by the degenerative reaction in the anterior roots of the corresponding side In the eosin-methylene blue stained sections the degenerative root changes are recognized chiefly by the extensive proliferation of the neurilemmal nuclei, the numerous clear areas indicative of the loss of axis cylinders and myelin sheaths, and often by the presence of fat-laden mononuclear phagocytes When monkeys are sacrificed during the first few days after nerve cell destruction has occurred, none of these changes are seen in the preparations of the nerve roots fixed and stained

in this manner It is only later, when the axis cylinders and myelin sheaths of the corresponding, destroyed neurons have finally degenerated and the sheaths of Schwann and the phagocytic cells have responded, that the picture described above is most easily recognized (see Figs 6, 7 and 8) The condition of the nerve roots is thus not only an important index to the loss of nerve cells but also to the chronicity of the lesion, in dicating that the process has taken place a considerable time before the monkeys were sacrificed and that we were not dealing with animals that had very long incubation periods and would have developed paralysis if they had been allowed to live longer When the loss of nerve cells is massive as shown in Figs 3, 4, and 5 one does not need to be convinced of it by finding the degenerative reaction in the corresponding nerve roots There are many instances, however, where the number of neurons affected is much smaller and one cannot be certain that there has been a loss of nerve cells without finding the degenerative reaction in the corresponding roots

These pathological changes in the spinal cord, varying in degree and extent, were present in all the cases in which a diagnosis of non paralytic poliomyelitis was made on histological grounds alone These changes were sometimes limited to one level of the cord but more often they could be found at several levels, including the lumbar, thoracic, and cervical regions In addition to the lesions in the spinal cord there were frequently perivascular cuffing and interstitial and focal glial infiltration in the sub stance of the medulla, hypothalamus, and thalamus The reverse, however, was rare and a histological diagnosis of non paralytic poliomyelitis was not considered to be warranted without evidence of neuronal lesions in the spinal cord There was one exception to this rule, in which poliomyelitis virus was, nevertheless, isolated on passage It is well known that in typical paralytic poliomyelitis in monkeys the virus is most readily isolated during the first few days after the onset of paralysis, and that after a period of 2 weeks the virus is only rarely found even in animals infected with monkey adapted virus It was, therefore, not expected that in these monkeys which were inoculated with human virus and sacrificed at a time when the lesions were already chronic, virus could be readily demonstrated by pas sage Passage was, however, attempted in a number of instances because it was desirable to obtain additional proof of the true poliomyelitic nature of the lesions and also to establish whether or not virus from a monkey with non paralytic poliomyelitis could produce the typical paralytic disease in other monkeys and thus prove that the host played a definite rôle in determining the non paralytic nature of the infection

The spinal cord and medulla of each of 4 non paralytic poliomyelitis monkeys were passaged into each of 4 new monkeys with negative results Using single monkeys however, we have obtained irregular results even when the nervous tissue of frankly paralyzed monkeys was employed for passage Multiple monkeys were used in sub-

sequent attempts (Table I), and positive passage was obtained in 2 instances with the development of typical paralytic poliomyelitis It may be of interest to describe the 2 animals whose nervous tissue yielded the virus Monkey 1-39 was sacrificed 36 days after intracerebral and intraabdominal inoculation with a pool of the lungs, liver, spleen, and kidneys from a case of human poliomyelitis Although nothing abnormal was observed during life, the following lesions were found in the nervous system of this monkey In the thalamic region two sections revealed multiple foci of interstitial glial

TABLE I

Passage of Nervous Tissue from Monkeys with Non-Paralytic Poliomyelitis

Tissue from Monkey No	Passaged into Monkey No	Result
5-6	2-76	Negative
1-12	2-78	"
1-09	2-71	"
1-46	2-69	"
3 04	3-95	"
	3 96	"
1-39	2 65	Typical paralytic poliomyelitis
	2-66	Negative
1-40	2 67	"
	2-68	"
Pool of 1-10, 1 60, 2-43	4-03	"
	4-04	"
	4-05	"
3-14	3 97	Typical paralytic poliomyelitis (subsequent passage also positive)
Atypical—lesions in medulla, none in 6 levels of spinal cord Medulla passaged	4-06	Negative

infiltration and perivascular cuffing distributed chiefly in the tuber cinereum, thalamus, and globus pallidus In the medulla, midcervical, and lower cervical regions of the spinal cord there were occasional foci of interstitial glial infiltration and marked perivascular cuffing In the midthoracic level there were present in addition to these changes distinct glial nodules indicative of previous neuronophagia, in the upper lumbar, midlumbar, and sacral levels of the cord distinct outfall of neurons in the anterior and posterior horns with well-advanced degenerative changes in the roots were present in addition to interstitial infiltration and marked perivascular cuffing It is evident, therefore, that the infection was well past its acute phase when the monkey was sacrificed, and the isolation of virus in this case indicates that while there was obviously some sort of equilibrium between the virus and the host which prevented involvement of enough cells to produce

the paralytic disease, the virus was not destroyed in the process The other monkey (3 14) that yielded the virus was especially interesting because it did not exhibit the pathological changes which in our opinion, were necessary for a histological diagnosis of non paralytic poliomyelitis It exhibited no abnormal clinical signs and was sacrificed 35 days after intracerebral inoculation of the abdominal sympathetic ganglia from a case of human poliomyelitis Histologically 6 levels of the spinal cord presented no sign of outfall of nerve cells, no degenerative reaction in the ventral or dorsal roots and no interstitial infiltration or perivascular cuffing in one section of the medulla however, there was some glial infiltration in the reticular substance of one side and in the floor of the 4th ventricle on the other side, associated with perivascular cuffing of several vessels 1 of 2 monkeys in which the glycerol preserved medulla was passaged, developed typical flaccid paralysis of the extremities and the usual characteristic lesions of poliomyelitis were present in the spinal cord Further successful passage of this virus was accomplished and it was shown to be non pathogenic for mice

Transitory Paralytic Poliomyelitis and the Question of Partial, Reversible Damage to Neurons —The term "transitory paralysis," as used here, refers to a condition in which distinct though usually partial, paralysis of one or more extremities is present for half a day to about 2 days and then disappears Although our monkeys were not always kept sufficiently long to permit detection of this condition, it has been encountered 5 times among 60 paralyzed animals We were interested in determining, if possible, the pathological basis of the transitory paralytic disease and whether or not the rapid halt to progression as well as the regression of paralysis was associated with a disappearance of virus

The temperatures and clinical course of 4 of these monkeys are shown in Chart 3 The first animal (8 2) is especially interesting because of the extremely short duration of paralysis, which might have been missed if the monkey's course had not suggested frequent observation This monkey had been given 5 nasal instillations of an untreated stool suspension and one intraabdominal injection of an ether treated suspension of the same material When its temperature had fallen from 106 8–106 5° on the 14th and 15th days to 102 8°F on the 16th day, it was carefully exercised in the morning but no paralysis or weakness was noted In the afternoon of the same day (16th) there was definite partial paralysis of both lower extremities, while on the morning of the next day (17th) the monkey could climb again with no definitely discernible paralysis It was active and well on the 18th and on the 19th day when it was sacrificed Histologically, typical lesions were present, but the majority of nerve cells, some of which showed degenerative changes were intact, and some levels of the spinal cord appeared entirely normal Passage of cord from this animal produced typical prostrating paralytic poliomyelitis in another monkey The pathological changes in the other 3 monkeys shown in Chart 3 were more extensive in that in some regions of the spinal cord more than half of the anterior horn nerve cells had undergone necrosis and neuronophagia

It seems clear from the histological studies on these monkeys as well as on those with non paralytic poliomyelitis, that an appreciable number

of nerve cells must be destroyed for paralysis to become clinically apparent When, therefore, paralysis regresses within a day or two of its appearance the question arises whether some of the nerve cells which had been damaged by the virus recover or whether the remaining cells have become sufficient for apparently normal function It is obviously impossible to follow the fate of the same cell or group of cells under these conditions of study, but the numerous instances in which nerve cells have been found in a partially

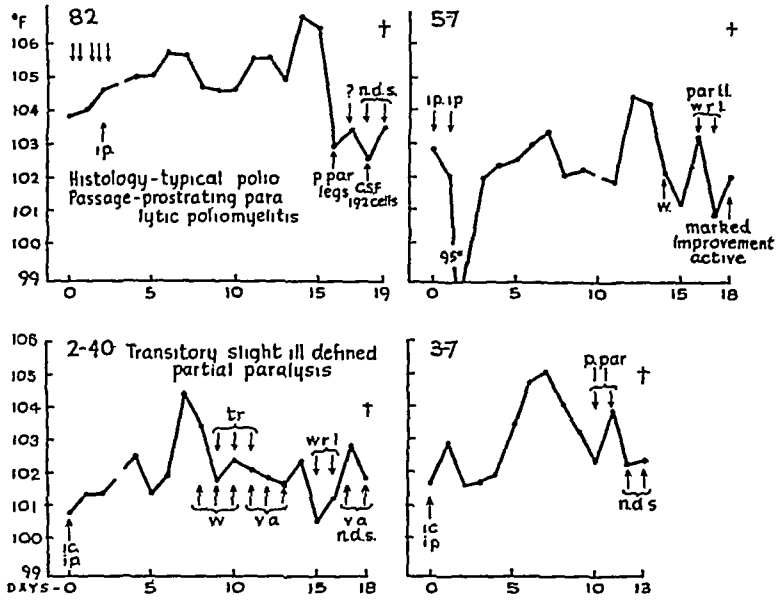


CHART 3 *Rhesus* monkeys with transitory paralytic poliomyelitis

damaged state suggest that a cell attacked by poliomyelitis virus perhaps need not invariably be irreversibly damaged

Fig 9 shows part of an anterior horn from monkey 3-7 which was sacrificed 2 days after regression of paralysis One can see in it, side by side, 1 or 2 normal cells (Fig 11), at least 4 cells with complete chromatolysis and typical acidophilic intranuclear inclusions (Figs 12 and 13), and several foci of neuronophagia (Fig 14) which are no longer acute in character, i e , they are made up of glial cells rather than polymorphonuclear leucocytes (see Figs 17 to 21 for comparison) In monkeys with non-paralytic poliomyelitis, sacrificed relatively early, one can see large numbers of cells with marginated Nissl substance and eccentric nuclei (Figs 2, 15, and 16) side by side with other changes indicating that the process is well past the acute phase The latter type of cytologic change has also been observed in some monkeys inoculated with human virus and sacrificed in the first few days after the onset of partial, non-progressing, though not regressing, paralysis In monkeys with non-paralytic or transitory, paralytic poliomyelitis sacri-

ficed approximately 2 weeks or more after the acute phase no such cells have been found the nerve cells surrounding glial foci or areas from which cells have obviously disappeared look quite normal (Fig 10) It would appear, therefore that either the degenerated cells and cells with intranuclear inclusions seen during the early stages of recovery are finally completely destroyed or else that they become normal again While it is clearly impossible to establish which of the two events occur, it is known that cells which retain their nuclei can recover and certainly cells in which the degenerative change shown in Fig 16 has been produced by other means, *e g* cutting peripheral nerves, etc can return to normal Nerve cells attacked by poliomyelitis virus that has been thoroughly adapted to the monkey (M V virus) as a rule pass through the stages indicated in Figs 17 to 21, beginning with chromatolysis and the appearance of acidophilic, intranuclear inclusions (usually seen when monkeys are sacrificed about a day before the onset of paralysis and rarely thereafter), and progressing to complete acidophilic necrosis and phagocytosis by polymorphonuclear leucocytes The presence of cells, such as those shown in Figs 12, 13, 15, and 16, in monkeys inoculated with human or first passage virus at a time when the host has clearly been able to check the further activity of the virus, suggests that the course of events depicted in Figs 17 to 21 may perhaps not be completed when a certain equilibrium has been achieved between the host and the virus

DISCUSSION

In the present study evidence has been brought forth that non paralytic infection can occur in *rhesus* monkeys inoculated with human or first passage poliomyelitis virus It was observed that while no reliable clinical or laboratory criteria were available, the diagnosis could be made with certainty when definite evidence of neuronal destruction in the spinal cord was found on histological examination of the nervous system The results of this study further indicate that in the absence of such changes in the spinal cord, suspicious lesions (*e g*, glial aggregates) in the medulla or elsewhere in the nervous system, though rarely present without simultaneous involvement of the cord, do not warrant the diagnosis of non paralytic poliomyelitis unless the virus can be isolated from the tissues The agent thus isolated must, however, produce the typical paralytic disease with necrosis of nerve cells and neuronophagia in the spinal cord in other monkeys, and not merely vascular or other vague infiltrative lesions For there is as yet no evidence that there exist strains of poliomyelitis virus without the cardinal property of producing neuronal lesions in the spinal cord and distinct flaccid paralysis The occurrence of non paralytic or transitory paralytic types of poliomyelitis in monkeys was found to depend upon the ability of the host to check the activity of the virus or to achieve an equilibrium with it, since it has been possible to produce the typical paralytic and prostrating type of disease by transferring tissue from these animals to other monkeys

In a recent report, Kling (7) maintained that in monkeys inoculated with

human tissues or drinking water there occur "abortive" or "mild paralytic" attacks of poliomyelitis in which there is no evidence of neuronal damage but only cellular infiltration in the meningeal vessels and choroid plexus On passage of the nervous tissue of such monkeys, "paresis" but not paralysis was observed, and there were again no neuronal lesions but only the vascular changes Although no evidence was obtained that these lesions were caused by poliomyelitis virus, he suggested that he was dealing with a type of poliomyelitis virus that caused no neuronal destruction We have observed the lesions described and illustrated by Kling in more than 50 per cent of our monkeys in which a diagnosis of poliomyelitis could not be made according to our criteria These lesions were also present in 30 of 44 monkeys inoculated with monkey tissues in which virus was never demonstrated We have seen a glial nodule in the medulla, perivascular cuffing, and cellular infiltration near the ventricles in uninoculated monkeys Furthermore, several attempts to passage nervous tissue showing these vascular lesions produced no apparent disease in other monkeys, and the nervous system of these passage animals was either free of similar lesions or contained them in the same ratio found in other monkeys We have as yet been unable to demonstrate any virus or other infectious agent pathogenic for monkeys, guinea pigs, or mice in the nervous tissues exhibiting these vascular lesions That rabbits and mice spontaneously exhibit in their nervous system similar changes, which must be guarded against when these animals are used in experimental work, is well known, and it is our impression that the same obtains for monkeys

The demonstration in the present study that monkeys do not need all their anterior horn nerve cells for apparently normal function, and that under certain conditions the host may achieve an equilibrium with the virus before a sufficient number of nerve cells is destroyed to produce paralysis, has a definite bearing on our interpretation of abortive and non-paralytic poliomyelitis in man It shows, for example, that the progression of virus need not necessarily be halted before it reaches the spinal cord for the infection to be non-paralytic, as was suggested in one hypothesis (9) It is also important to note that the evidence indicates not only that the virus can reach and attack the spinal cord in a non-paralytic infection but also that it may persist for a considerable period In this respect one cannot help but wonder whether or not certain activities or conditions may not be capable of upsetting such an equilibrium, and one recalls the frequency with which certain cases of human, paralytic poliomyelitis occur within about 24 hours of severe exertion or excessive exercise The short interval between the exertion and the onset of paralysis suggests that the virus must

have already been present in the central nervous system at the time, and the question naturally arises whether or not an infection that might have remained non paralytic could thus be changed into the paralytic disease by a disturbance in the equilibrium which may exist between the host and the virus

The phenomenon of transitory paralysis which is not unknown in human poliomyelitis has in earlier days been explained on the belief that edema and inflammatory exudate temporarily interfered with the function of the nerve cells Histological study of this condition in monkeys, however, offers no support for such a hypothesis and suggests rather that it may depend in part on the fact that apparently normal function can be carried on with less than the normal number of nerve cells, and in part on the possibility that not all nerve cells attacked by the virus are irreversibly damaged

SUMMARY AND CONCLUSIONS

1 The occurrence of non paralytic poliomyelitis in monkeys inoculated with human or first passage virus was proved by histological examination of the nervous system and by isolation of the virus

2 The non paralytic infection was almost invariably associated with the destruction of an appreciable number of nerve cells in the spinal cord, and failure of the process to progress seemed to depend upon an equilibrium between the host and the virus, in which the latter occasionally persisted in an active state since it could produce the typical paralytic disease on passage to other monkeys

3 While there were no reliable clinical or laboratory criteria, the diagnosis of non paralytic poliomyelitis was made when the following changes were found in the spinal cord (*a*) outfall of neurons confirmed by the presence of the reaction of degeneration in the nerve roots, and (*b*) foci of glial infiltration and perivascular cuffing in the gray matter

4 Anterior horn cells showing diffuse chromatolysis and acidophilic, intranuclear inclusions were present 2 days after disappearance of paralysis of short duration, and nerve cells with marginated Nissl substance and eccentric nuclei were found side by side with obviously older lesions in monkeys with non paralytic poliomyelitis These cytologic changes were not present in monkeys sacrificed in still later stages of the disease

5 The transitory character of the paralysis in some monkeys may depend in part on the fact that apparently normal function can be carried on with less than the normal number of nerve cells and in part on the probable, but not proved, possibility that not all nerve cells attacked by poliomyelitis virus are irreversibly damaged

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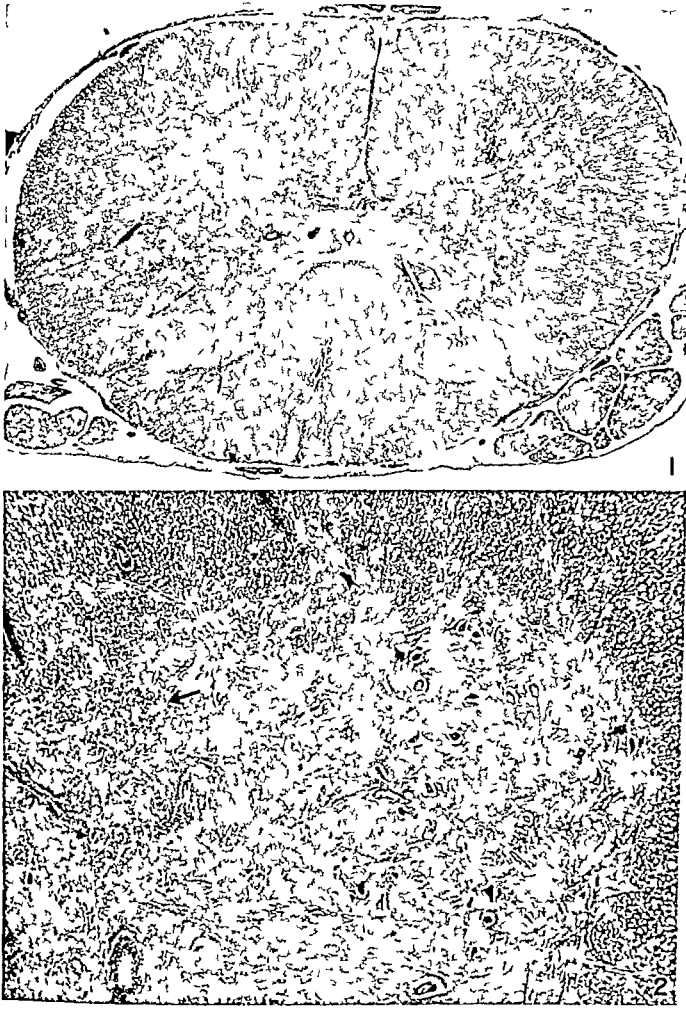
EXPLANATION OF PLATES

PLATE 35

FIG 1 Upper lumbar cord of monkey (5-6) with non-paralytic poliomyelitis × 19

FIG 2 Same, left anterior and lateral horns Note outfall of nerve cells and cellular infiltration in lateral horn, and anterior horn cells with marginated Nissl substance and eccentric nuclei × 65



(Sabin and Ward Poli myel t n monkeys)

PLATE 36

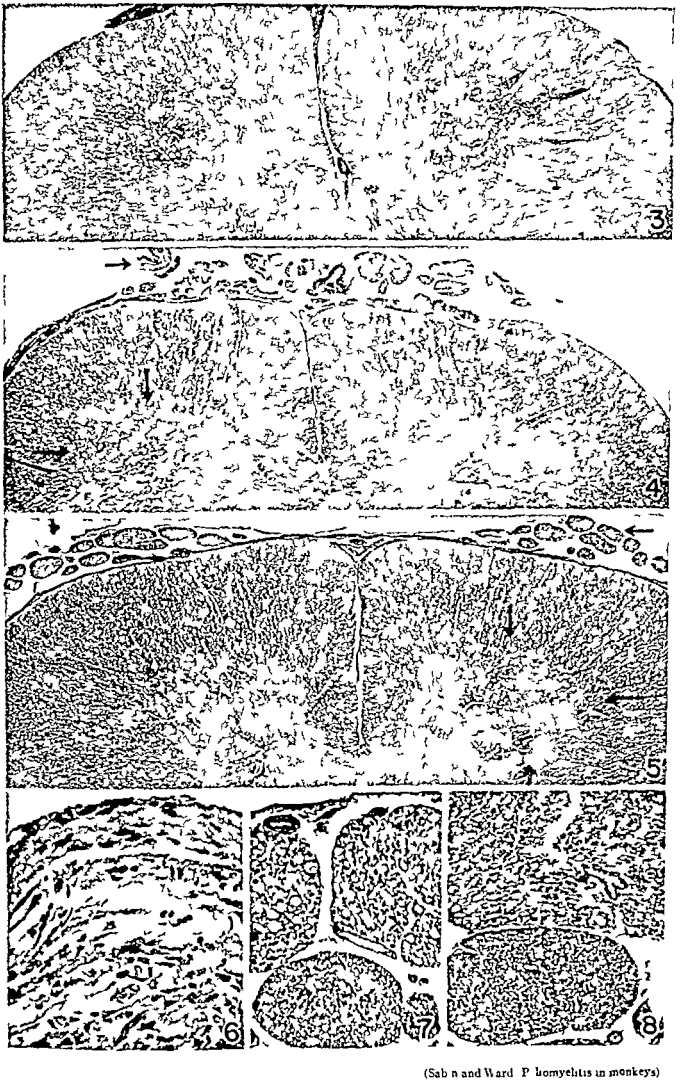
FIG 3 Monkey (7-6) with non-paralytic poliomyelitis Note destruction of most of the anterior horn on the right side × 16

FIG 4 Monkey (1-10) with non-paralytic poliomyelitis sacrificed at later stage Arrows point to outfall of neurons in anterior horn on the left, and to roots showing reaction of degeneration For greater magnification see Fig 6 × 20

FIG 5 Monkey (1-46) with non-paralytic poliomyelitis Arrows point to outfall of neurons in the anterior horn on the right and to degenerated nerve roots on the same side Note paucity of reaction in site from which neurons have disappeared × 20

FIG 6 Nerve root showing reaction of degeneration × 270

FIGS 7 and 8 Same The roots in the lower portion of each figure are normal Fig 7, × 103 Fig 8, × 68



(Sabin and Ward Poliomyelitis in monkeys)

PLATE 37

FIG 9 Anterior horn in spinal cord of monkey (3-7) with transitory paralysis, sacrificed 2 days after disappearance of paralysis Black arrows point to foci of neuronophagia (see Fig 14), and white arrows to nerve cells with acidophilic, intranuclear inclusions (Figs 12 and 13) × 68

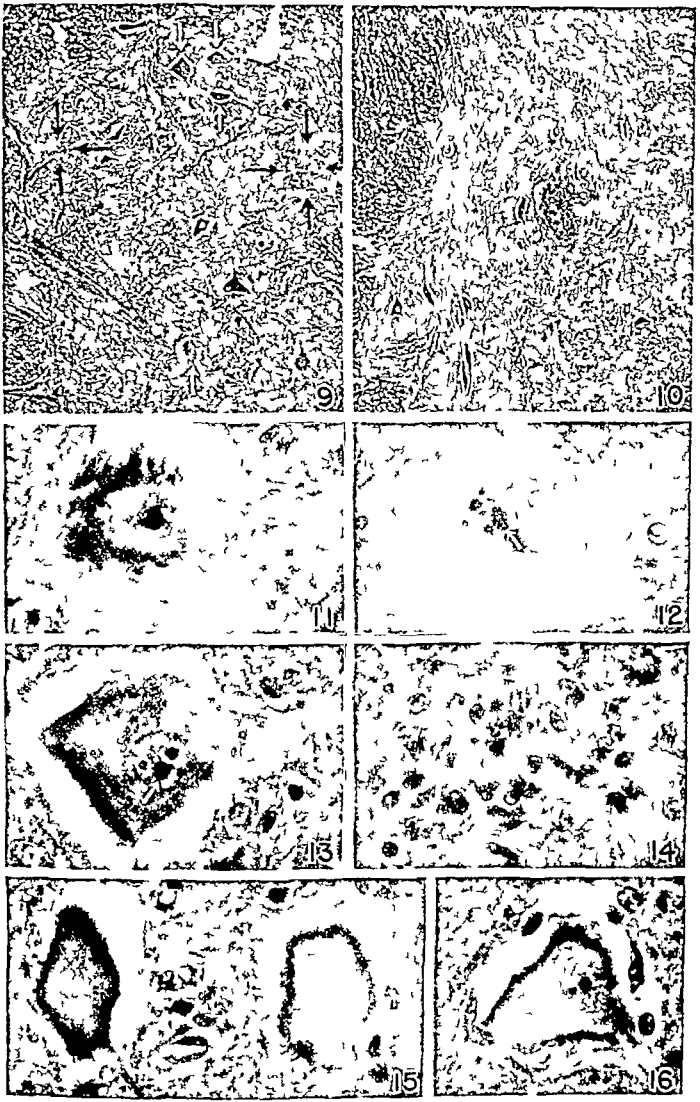
FIG 10 Anterior horn in spinal cord of monkey (1-40) with non-paralytic poliomyelitis, sacrificed 36 days after inoculation Note foci of cellular infiltration indicative of previous loss of nerve cells, and the normal appearance of the remaining nerve cells × 68

FIG 11 Apparently normal cell from Fig 9 × 640

FIGS 12 and 13 Cells showing diffuse chromatolysis and acidophilic intranuclear inclusions from Fig 9 Arrows point to basophilic nucleoli—other intranuclear, sharply outlined, bodies are acidophilic inclusions × 640

FIG 14 Focus of neuronophagia from Fig 9 Note that it is made up chiefly of glial cells × 640

FIGS 15 and 16 Anterior horn cells from Fig 2 showing margination of Nissl substance and eccentricity of nucleus × 640



(Sab nd Ward P l my l t s i monkeys)

Plate 38

Figs 17 to 21 depict the fate of an anterior horn cell attacked by monkey adapted poliomyelitis virus They were prepared in the course of an unpublished study on the pathology of experimental poliomyelitis produced by nasal instillation of M V virus, by one of us while at the Laboratories of The Rockefeller Institute for Medical Research The photographs were taken by Mr Joseph B Haulenbeek

Fig 17 Normal anterior horn cell representative of condition of the nerve cells in the spinal cord about 3 days before the onset of paralysis × 1000

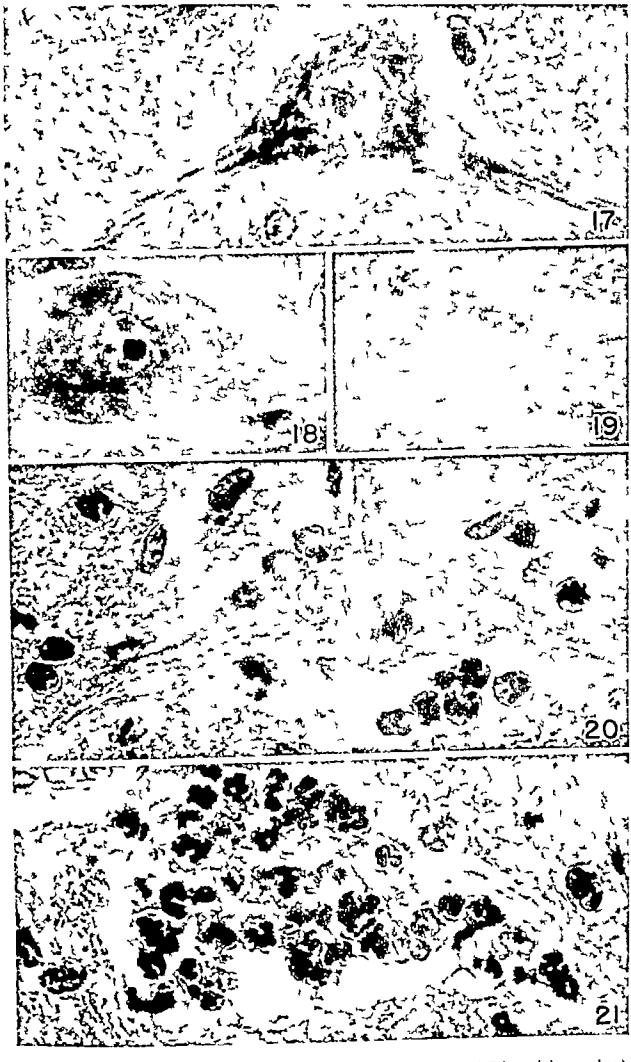
Fig 18 Anterior horn cell showing diffuse chromatolysis and three acidophilic, intranuclear inclusions, found almost exclusively in monkeys sacrificed the day before onset of paralysis × 1000

Fig 19 Complete acidophilic necrosis of anterior horn cell × 1000

Fig 20 Polymorphonuclear leucocytes invading necrotic cell × 1000

Fig 21 Neuronophagocytosis by polymorphonuclear leucocytes × 1000

The stages shown in Figs 19 and 20 predominate on the day before paralysis while that in Fig 21 is more prevalent during the first day of paralysis This timing was made possible by the fact that the virus used was so well adapted that it not only regularly produced infection by the nasal route but also that paralysis appeared on or about the 7th day in almost all inoculated monkeys



(S b and Ward P I my litis in monkeys)

THE NATURAL HISTORY OF HUMAN POLIOMYELITIS

I Distribution of Virus in Nervous and Non Nervous Tissues*

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The distribution of virus in the tissues of an infected host is frequently, if not always, a key to the nature of the disease, and experimental studies with a number of neurotropic viruses in recent years have shown that a proper investigation of such distribution can point not only to the site from which the virus invades the nervous system but also to its mode of spread and elimination

Our present concepts of the nature of human poliomyelitis are based (*a*) on certain limited investigations of human tissues made for the most part 25 to 30 years ago when the virus was first recognized and the criteria for its identification were not always rigid, and (*b*) on the behavior of the virus in experimental animals, different species of which appear to behave quite differently (1, 2) The scattered existing data on the infectivity of human tissues include tests on the spinal cord medulla and cerebrospinal fluid the lymph nodes including the tonsils and pharyngeal tissue, the nasal mucosa, the blood and viscera and as regards secretions and excretions, nasopharyngeal washings and intestinal contents or stools No virus has been demonstrated in the cerebrospinal fluid, the blood or the viscera Earlier reports of the presence of virus in the lymph nodes, particularly those of the mesentery, could not be corroborated by numerous subsequent tests and the explanation was offered that in the early work the lymph nodes might have been contaminated by admixture with positive tissues (3) Recently, however, the occasional presence of virus in human cervical and mesenteric lymph nodes has again been reported, although the criteria used for identifying the virus in some instances are open to question (4, 5) The early repeated positive results with the tonsils and attached pharyngeal mucosa (6–8) have not been reinvestigated in recent years and it has re

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The material in this communication has been presented in part at the 1940 meeting of The Society of American Bacteriologists (*J Bact* 1941, **41**, 49)

mained a question whether the virus was present in the tonsils or in the pharyngeal mucosa The nasal mucosa has been tested in only three cases and the virus was reported to be present in one (8) The numerous tests with nasopharyngeal washings through the years have yielded a certain number of positive results, although it has never been made clear whether the virus came from the nose or from the mouth and pharynx The infectivity of the intestinal contents which had been denied for many years was established beyond doubt in more recent work, although the source of the virus thus eliminated remained unknown In no instance, however, have all the tissues and secretions or excretions upon which tests are recorded been studied simultaneously in the same case It was clear, therefore, that the existing data, even including the controversial studies, were insufficient for formulating any idea of the essential nature of human poliomyelitis

Plan of Study

The plan of the present sudy was to search for the virus of poliomyelitis in a sufficiently large number of properly selected tissues to enable one to determine whether or not its distribution follows a distinct pattern Although past experience indicated that the primary transmission of the virus from human tissues to monkeys is not always readily accomplished, it nevertheless appeared possible that if all the selected tissues were obtained from each of six or more cases of human poliomyelitis and tested in the same manner, each specific structure would be inoculated into monkeys a sufficient number of times to give at least an indication of the presence or absence of virus in it The selection of tissues for these tests was determined by their capacity to indicate (*a*) whether or not a certain system was affected, (*b*) what might be the centrifugal or centripetal pathways pursued by the virus outside the central nervous system, and (*c*) whether at the time of death the virus is distributed indiscriminately throughout the central nervous system or is present in appreciable amounts in some areas and not in others in accord with a definite pattern

Table I lists the tissues selected for study and the order in which they were removed from the body The pharyngeal mucosa with or without the tonsils was tested not only because in the work of 25 to 30 years ago the virus was reported most regularly as being present in the tonsils, but also because the pharynx is part of the alimentary tract and the tonsils are part of the cervical lymphatics Practically all the superficial and deep cervical lymph nodes, and the mesenteric lymph nodes were dissected out and tested because the presence or absence of virus in them is still a matter of controversy The axillary and inguinal lymph nodes were included for control in order to determine whether the virus may possibly be widely distributed in the lymphatic system and be responsible for the pathological changes which are so frequently encountered in it The liver, spleen, kidneys, and lungs were selected as an index to the generalization of the virus, but we now think that it was a mistake to include the lungs in the pool because of the frequency of aspiration of material from the alimentary tract in terminal cases The

suprarenals were tested chiefly because they contain so many neurons of the autonomic system which might be involved in case appreciable centrifugal spread of virus occurred The entire submaxillary and part of the parotid salivary glands were obtained to determine whether or not the virus of poliomyelitis, like that of rabies, may spread to them centrifugally and whether or not the saliva could thus become one means of elimination 12 to 18 inches of ileum near the ileocecal junction and of descending colon with their respective contents were tied off and excised The intestinal contents and the thoroughly washed walls were tested separately The presence or absence of virus in the wall of the gut with its nerve cells of the submucous and myenteric parasympathetic plexuses (Meissner s and Auerbach s) would throw considerable light not only on the origin of virus in the stools, but taken together with the other data also on the possible site or sites from which the nervous system may be invaded The cervical and abdominal sympathetic ganglia were tested to indicate whether or not centripetal or centrifugal spread of virus occurs along their course The nasal mucosa, both the nervous, olfactory, and the respiratory portions was included to determine whether or not it is a site

TABLE I

Human Poliomyelitis Necropsies

Tissues Selected for Study and Order of Their Removal

1 Pharyngeal mucosa with or without tonsils
2 Axillary and inguinal lymph nodes
3 Suprarenals
4 Abdominal sympathetic ganglia
5 Liver spleen kidneys lungs
6 Mesenteric lymph nodes
7 Ileum (near ileocecal junction) and contents
8 Descending colon and contents
9 Cervical lymph nodes
10 Salivary glands (submaxillary and parotid)
11 Superior cervical sympathetic ganglia
12 Olfactory bulbs
13 Anterior perforated substance and adjacent corpus striatum
14 Diencephalon
15 Pons and medulla
16 Anterior frontal and occipital portions of neopallial cortex
17 Motor cortex
18 Mesencephalon
19 Spinal cord
20 Nasal mucosa (olfactory and respiratory)

from which infective virus may be eliminated In the central nervous system tests on the olfactory bulbs and anterior perforated substance were expected to throw light on whether or not the virus invades by the olfactory pathway This deduction however, would be possible only if it were found that there was no generalized diffusion of the virus involving areas such as the anterior frontal and occipital portions of the neopallial cortex in which no neuronal lesions are found The diencephalon mesencephalon motor cortex, pons and medulla, and spinal cord were tested to check the frequency with which the virus may be isolated from regions in which the lesions are usually localized

Since the olfactory pathway has received special consideration as a possible portal of entry of the virus and since the pathological changes observed in the olfactory bulbs of monkeys infected by the nasal route (9) were not found in human olfactory bulbs (10) it was especially desirable to establish whether or not they contained the virus Consequently it was planned to study several additional cases in which only the olfactory bulbs and spinal cord would be investigated

Methods

In preceding studies on human poliomyelitis tissues have usually been sent to the laboratory (occasionally all mixed together in a single bottle) by pathologists unaware

of the precautions that must be observed Our material was derived from necropsies carried out by ourselves under relatively aseptic conditions and with a sufficient number of sterile instruments to permit the individual handling of each tissue or group of tissues to be tested The necropsies were performed in Indiana, Ohio, and West Virginia where a moderate epidemic of poliomyelitis occurred in 1940 In some instances it was possible to begin the necropsy within a few hours after death while in others longer delays were unavoidable

The skin was prepared with iodine and alcohol The usual incision was used for obtaining the thoracic and abdominal tissues To expose the salivary glands and other structures of the neck, the original incision ending on the shoulder over the acromion process was carried backwards along the upper edge of the scapula which permitted reflection of the skin of the neck up to the mandible To obtain the abdominal sympathetic ganglia, the descending aorta was exposed at the level of the left suprarenal and the large celiac ganglia and associated plexuses were dissected, a small piece was occasionally sectioned for histological confirmation of the tissue No attempt was made to expose the skull aseptically However, after the scalp had been reflected, the area was washed with alcohol and the bone, through which the saw was to pass, was cleared of periosteum and similarly treated with alcohol A sterile saw was used but great care was taken not to incise the dura After removal of the calvarium, all further work was done with sterile rubber gloves since the brain had to be removed manually In order to avoid tearing or maceration of the olfactory bulbs, the frontal poles were lifted with a sterile spatula and the olfactory tracts were cut before the brain was removed from the cranial cavity The brain was placed on sterile towels and the various portions, dissected out with separate instruments, were put into individual bottles The nasal mucosa was obtained last through an intracranial approach after stripping the dura mater and thorough cleansing with alcohol of the bones forming the roof of the nose The order in which the tissues were removed, as indicated in Table I, was arrived at after some trial and error and was chosen chiefly because it permitted the dissection of the various structures in relatively bloodless fields

In the beginning, the tissues were kept in 50 per cent, buffered glycerol, transported to the laboratory in the frozen state packed in solid CO_2, and stored in the refrigerator at about 5°C until used Later on, however, the tissues (without glycerol) were frozen immediately after removal from the body and kept frozen with solid CO_2 until tested Although we now prefer the latter method, there was actually no difference in the results obtained with the tissues preserved by either procedure Representative sections of the nervous system (usually cervical, thoracic, and lumbar levels of the spinal cord and one level of the medulla to include the nuclei of the 9th and 10th cranial nerves) were obtained in each case for histological study to ascertain whether or not the pathological findings were in accord with the clinical diagnosis of poliomyelitis

Preparation of Tissues for Inoculation —With the exception of material such as nasal mucosa, pharyngeal wall, or tonsils, ileum, colon, and their respective contents, which are naturally contaminated, all the other tissues were prepared for inoculation as heavy suspensions in distilled water or physiologic salt solution, and the aseptic precautions which were observed in obtaining them were sufficiently adequate to prevent death of the animals from bacterial infection Both olfactory bulbs were ground without added abrasive and the total amount, usually 1 5 to 2 cc was injected intracerebrally (i c) With the exception of the spinal cord and medulla approximately 5 gm of each of the

other parts of the nervous system were used, unless as in the case of the diencephalon or anterior perforated substance, the entire specimen weighed less than 5 gm Whenever possible the nervous tissues were ground without abrasives, and the total milky suspensions after filtration through several layers of gauze were inoculated i c and intra abdominally (i p) The lymph nodes and salivary glands which frequently exceeded 10 to 15 gm were used *in toto* and were inoculated as slightly centrifuged 20 per cent suspensions Approximately 10 gm of the pool of viscera were used for preparing a 20 per cent suspension, all of which was inoculated into a single monkey The contaminated tissues were prepared differently The nasal mucosa and the pharyngeal mucosa with or without the tonsils were ground with sand and taken up in sufficient distilled water or saline to yield a 10 per cent suspension After horizontal centrifugation at about 2000 R P M for 10 minutes, the supernatant liquids were drawn off, mixed with 15 per cent of their volume of anaesthetic ether, shaken for 10 minutes, and left in the refrigerator overnight This was followed by horizontal centrifugation at 2000 R P M for 10 to 20 minutes, and the middle layer between the sediment and the fatty cake at the top was pipetted off and again centrifuged at the same speed for 30 to 60 minutes The final supernatant liquid was almost always sterile on blood agar and was used for i c and i p inoculation The original sediment before ether treatment was occasionally resuspended and used for nasal instillation in the same monkey which received the ether treated material The loops of intestine were opened in a large Petri dish and the contents separated from the wall with the aid of wooden tongue-depressors The wall was then cut in several pieces, shaken several times with fresh changes of 500 to 1000 cc of tap water and left to wash in running tap water until the wash water was quite clear the water was drained off and the intestinal wall was ready for use. As a rule, approximately 10 gm of the intestinal wall were ground with sand and enough distilled water or saline to make a 20 per cent suspension This suspension was then submitted to the same ether treatment and series of centrifugations which were described for the nasal mucosa and pharyngeal wall With the exception of the ileum and colon contents all tissues were inoculated i c and, whenever more than 4 cc. of suspension was available, i p as well The ileum and colon contents were weighed and, depending on the amount, 10 or 20 per cent suspensions were prepared, and the same procedure of etherization and centrifugation, described for the other contaminated tissues was followed here On several occasions it was necessary to repeat the process of etherization and centrifugation two or three times before it was possible to obtain material that would not kill the inoculated monkeys within 24 to 48 hours Since specimens so treated finally produced typical poliomyelitis in monkeys the procedure is obviously not too harmful for the virus The sediments from the first centrifugation before etherization were resuspended and used for nasal instillation (repeated daily for 6 to 10 days) in the same monkeys which received the ether treated material i p and occasionally i c. Whenever enough material was available two i p inoculations of 20 cc each were given 24 or 48 hours apart

Animals and Observations —Since *Macacus rhesus* monkeys were the only ones available in sufficient numbers for this study, they were used in most of the tests *Macacus cynomolgus* monkeys have been reported by several observers as being somewhat more susceptible They were therefore used for a series of tissues from two cases, although in a comparative test with the spinal cords from five human cases which failed to produce poliomyelitis in *rhesus* monkeys on first inoculation the *cynomolgus* did not prove to be superior in revealing the virus Intracerebral inoculations were made under local anes

thesia with novocain and adrenalin, because it was possible to give nasal instillations of contaminated material to such monkeys without deaths from pneumonia which occurred with great frequency among the animals that had had ether anesthesia Rectal temperatures were taken daily and the animals were exercised to permit the detection of even slight degrees of paralysis as reflected in abnormal running or climbing If the animals remained well during the first week or two they were frequently given a second intracerebral inoculation of the same material preserved in the refrigerator, and if they still developed no suggestive signs they were kept for 5 to 6 weeks after the first inoculation and were then killed for histological study As a rule, 6 levels of spinal cord (3 lumbar, 1 thoracic, and 2 cervical) and one level of medulla (through the 4th ventricle) were sectioned, although in certain instances other regions of the nervous system were also examined The olfactory bulbs and anterior perforated substance were included whenever nasal instillations had been given Care was taken to include the roots in the sections of the spinal cord, because their condition can be of great assistance in establishing a diagnosis of non-paralytic poliomyelitis (11) The remaining portions of the spinal cord and medulla were saved in 50 per cent buffered glycerol and passage was often resorted to when the diagnosis was in doubt The viscera were examined in every monkey and it is noteworthy that only one case of tuberculosis was encountered among the more than 200 animals used in this study The human spinal cord or medulla and pons suspensions were also injected i c and i p into 6 to 10 young mice The mice, however, remained well and had no immunity 2 months later to Armstrong's mouse passage virus (Lansing strain)

Criteria for Diagnosis of Experimental Poliomyelitis —When a monkey developed unmistakable flaccid paralysis of one or more extremities with characteristic neuronal necrosis, neuronophagia, and infiltrative lesions in the spinal cord, the diagnosis of experimental poliomyelitis was made without resorting to passage in each instance Positive passage was, however, obtained with one or more tissues in most cases, which taken together with the non-pathogenicity of the material for mice, was considered sufficient basis for diagnosing the presence of poliomyelitis virus in the human material In the preceding communication (11) we described the occurrence of non-paralytic poliomyelitis, established by histological examination, with typical lesions in the spinal cord in monkeys inoculated with human virus Although the diagnosis of non-paralytic poliomyelitis was made eleven times, it was not necessary to base any significant conclusion on that diagnosis alone, since in all but one instance, either the same tissue from other cases gave rise to typical paralysis or positive passage with the development of typical paralytic poliomyelitis was obtained Histologically the diagnosis of experimental poliomyelitis was not made without evidence of past or present neuronal lesions in the spinal cord, despite the one exception which we have encountered (11)

RESULTS

Eleven complete necropsies were performed in which all the tissues enumerated in Table I were obtained Two of these turned out not to be poliomyelitis on histological examination of the human spinal cord and medulla and the monkeys inoculated with these tissues remained well It is of interest to note with respect to the type of disease that may be clinically confused with poliomyelitis, that one of these cases was most likely

an osteomyelitis of the leg with a terminal pyogenic pleuritis, pneumonia, pericarditis, and focal myocardial abscesses, and the other was an infectious polyneuritis (neuronitis) with typical lesions in the nerve roots In two additional cases of poliomyelitis only a limited number of tissues were obtained

To permit a critical analysis of the results it seems desirable to present in brief form the significant protocols including the history of the patient, the essential pathological findings in the human nervous system, and the type of experimental disease produced in the monkeys inoculated with the various tissues The inoculations resulting in the demonstration of polio myelitis virus in certain tissues of the first seven cases are summarized in Table II

Case 1 (*Ohio*) —Art , 5 year old white boy Onset Aug 12, 1940, with headache, vomiting, and fever (101°F) Aug 13, vomiting relieved, fever 101 F Aug 14, a m , paralysis both arms left leg and face p m paralysis left intercostal muscles aphonia and dysphagia, died 8 30 p.m in respiratory failure

Body not in refrigerator necropsy begun 5½ hours after death Only remarkable gross finding was extreme enlargement of cervical lymph nodes and extreme softness and mushrooming of spinal cord Microscopically there was extensive neuronophagia and cuffing throughout the spinal cord more marked on one side than on the other at the lumbar level, and with complete destruction of almost all the nerve cells in the thoracic and cervical levels Similar focal lesions were present in the medulla but less marked than in the spinal cord

Case 2 (*Ohio*) —Wil 8 year old white boy Reported not to have been feeling well and to have had some fever for 2 weeks before onset on Sept 7, 1940, with abdominal pain vomiting and headache Sept 8 vomiting and fever Sept 9 nasal voice and dysphagia on admission to hospital temperature 102°, stiff neck, questionable right facial paralysis, palatal paralysis, hoarse nasal voice to no voice at all, no paralysis of the extremities or loss of deep tendon reflexes pleocytosis of 500 cells, mostly lymphocytes *Sept 10, temperature 103 extremely restless* and general condition worse, died 9 30 p.m

Body in refrigerator, necropsy begun 8½ hours after death The marked enlargement and edema in the gross of all the lymph nodes—cervical, mesenteric axillary, and inguinal—and small white foci in parts of the liver were the remarkable findings Microscopically one level of the medulla presented heavy cuffing and extensive neuronophagia affecting chiefly the nucleus of the hypoglossal nerve on one side and the nuclei ambigui and reticular substance of both sides *Despite the* absence of obvious paralysis of the extremities and the presence of *the reflexes a section through* the midcervical region of the spinal cord revealed neuronophagia of about half the number of the anterior horn cells and heavy cuffing while sections through the thoracic and lumbar levels also showed focal neuronophagia and heavy cuffing There was necrosis slight polymorphonuclear cell infiltration and proliferation affecting chiefly the reticulum of all the lymphoid tissue in the nodes, spleen, and intestines The liver showed numerous areas of focal necrosis

TABLE II

Data on Tissues in Which Poliomyelitis Virus Was Demonstrated

Case No	Tissue	Dose and route	Monkey No	Result of inoculation				Passage
				Fever	Paralysis	Died or killed	Pathology	
		cc						
1	Spinal cord	2 i c 15 i p	Rh 1 23	0	6* (A + L)	K 6*	Typical	+ (1/1)
	Medulla and pons	2 i c 27 i p	Rh 1 58	0	14 (Wk 96 4)	K 14	"	
	Diencephalon	2 i c 30 i p	Rh 1 54	7 8*	8-12 (RF, L)	K 12	"	
	Motor cortex	2 i c 50 i p	Rh 1 56	7	8-10 (L)	K 10	"	-
	Ileum—contents	2 i c 3 5 i p	Rh 1 50	0	5 (Aph , Prostr)	D 6	"	+ (1/1) NP
	Ileum—washed wall	2 i c 45 i p	Rh 1-49	0	15 17 (LA)	K 17	"	0 (0/1)
	Pharyngeal + tonsils	2 i c (r15) 6 i p	Rh 1-46	0	0	K 39	NP	0 (0/1)
	Axillary + inguinal lymph nodes	2 i c (r16) 17 i p	Rh 1-40	irr	0	K 36	NP	0 (0/2)
	Lung + liver + spleen + kidney**	2 i c (r16) 40 i p	Rh 1 39	irr	0	K 36	NP	+ (1/2)
2	Spinal cord	2 i c 26 i p	Rh 1 91	0	11-12 (L)	K 12	Typical	+ (1/1)
	Medulla and pons	2 i c 20 i p	Rh 1-60	0	0	K 36	NP	
	Mesencephalon	2 i c 46 i p	Rh 2 16	8	10-11 (A)	K 11	Typical	0 (0/1)
	Diencephalon	2 i c 30 i p	Rh 2 17	0	13-17 (part RA, LA)	K 17	"	
	Motor cortex	2 i c 44 i p	Rh 2 18	0	15-19 (trans L)	K 31	"	
	Pharyngeal tissue	2 i c 17 i p 4 i n	Rh 2 24	0	11-13 (RF, N)	K 13	"	+ (1/1)

Legend —A, both arms, L, both legs, RA, right arm, LA, left arm, RL, right leg, LL, left leg, F, facial, N, neck, Aph , aphonia, Prostr , prostrate, Wk., weakness, part , partial, trans , transitory paralysis, Rh, *rhesus,* Cyn, *cynomolgus,* NP, non paralytic, r15, reinoculated on 15th day, i c , intracerebral, i p , intraabdominal, i n , intranasal, irr , temperature irregular

* Numerals refer to day of fever, paralysis, death, or sacrifice D, dead, K, killed

** *Case 1 Pooled Viscera* —When it was established that the virus was contained in the pooled viscera, an attempt was made to discover in which it was present The remaining pieces, all contained in one bottle of glycerol, were thoroughly washed in physiologic salt solution, and what was left of each organ was inoculated into separate *rhesus* monkeys as follows *lung*—2 cc , i c and 5 5 cc , i p with reinoculation i c of 2 cc 7 days later, *liver*—2 cc , i c , 12 cc , i p , and reinjection i c 7 days later, *kidney*—2 cc., i c , 18 cc , i p , and reinjection i c 7 days later All monkeys remained well and no histologic evidence of poliomyelitis was found

TABLE II—*Continued*

Case No	Tissue	Dose and route	Monkey No	Result of Inoculation				Passage
				Fever	Paralysis	Died or killed	Pathology	
		cc						
Case 2—*cont'd*	Ileum—contents‡	2 i.c. 21 i p 12 i.n	Rh 2 59	15 16	17 20 (part. RA L)	K 20	Typical	
	Descending colon—contents	20 i n 20 i p 6 i.p	Rh 2 50	8 9 15 16	18 19 (part A + L)	K 19	Typical (ol factory +)	
3	Spinal cord (cervical)	2 i.c 21 i p	Rh 1 97	8 9	9–11 (F Wk N part. RL)	K 11	Typical	+ (NP)
	Medulla and pons	2 i c 20 i p	Rh 1 27	0	13 15 (N A, L Prostr)	K 15		
	Mesencephalon	2 i c 43 i p	Rh 1-85	7	10 (RL part LL)	K 10		
	Diencephalon	2 i c 25 i p	Rh 1-84	8	9–11 (RF RL part LL)	K 11		
	Ileum—washed wall	2 i c 40 i p	Rh 1 78	0	0	K 35	0	
		2 i c 70 i p	Rh 2 55	20	21 22 (RL part LL)	K 22	Typical	+ (2/2)
	Descending colon—contents	12 i.p	Rh 1-81	0	14 15 (RF N RL part. LL + A)	K 15	Typical (ol factory—0)	+ (1/1)
4	Spinal cord	2 i.c 10 i p	Rh 1-09	13 14	0 Tr 15 16	K 21	Typical	0 (0/1)
	Medulla and pons	2 i c 10 i p	Rh 76	6–9	0 Wk RL 13 14	K 20		
	Motor cortex	2 i c 36 i.p	Rh 1 12	0	0	K 37	NP	0 (0/1)
	Mesencephalon	2 i c (r15) 26 i p (r15)	Rh 1 10	0	0	K 37	NP	
	Descending colon—contents	22 i p	Rh 20	12 13	14–16 (part A + L)	K 16	Typical (ol factory—0)	+ (NP)
5	Spinal cord	2 i.c 27 i p	Rh 1 93	0	0	K 35	0	
		2 i.c 26 i c	Rh 2-40	0	9–16 trans ?	K 18	Typical	
		2 i c. 26 i p	Cyn 2-39	0	15–19 (part RL Wk L)	K 19		

‡ *Case 2 Ileum—Contents*—8 gm. contents suspended in 100 cc. distilled water etherized. 30 colonies per cc after etherization Rh 2 25—2 cc i c. and 1 cc. of untreated sediment in each nostril—dead following morning with bacterial meningitis Rh 2 26—20 cc. i p on Oct 13 and same on Oct. 15 with nasal instillations on 3 successive days—dead 6th day with acute pentonitis Remaining material etherized second time and 0 1 cc. yielded no growth inoculated into Rh 2 59

TABLE II—*Concluded*

Case No	Tissue	Dose and route	Monkey No	Result of inoculation				Passage
				Fever	Paralysis	Died or killed	Pathology	
		cc						
Case 5—*cont'd*	Pharyngeal + tonsils	2 i c 35 i p	Rh 3-04	11 21, 23	0	K 35	NP	0 (0/2)
	Abdominal sympathetic plexus§	2 i c	Rh 3 14	irr	0	K 35	Only in medulla	+ (1/2)
	Descending colon—contents	12 i n 20 i p 10 i p	Rh 3 21	irr	20-24 (part LL)	K 24	Typical (olfactory +)	
6	Spinal cord (cervical)	2 i c 21 i p	Rh 1 96	0	0	K 35	0	
	Various regions	2 i c 23 i p	Rh 2-43	irr	0	K 36	NP	
		2 i c 23 i p	Cyn 2-44	9 10	13-16(RL)	K 16	Typical	
	Mesencephalon	2 i c (r7) 32 i p	Rh 2 99	6, 9	9-11 (L, part A)	K 11	"	
	Diencephalon	2 i c (r7) 28 i p	Cyn 2 97	6	9-10 (L)	K 10	"	+ (3/3)
	Pharyngeal + tonsils	2 i c 15 i p	Cyn 2-84	irr	8-12 (L RA, part LA)	K 12	"	
	Ileum—washed wall	2 i c 36 i p	Cyn 2 88	irr	11-12 (F L, Wk A)	K 12	"	+ (2/2)
	Descending colon—washed wall	2 i c 40 i p	Cyn 2-86	14	17-18 (A + L)	K 18	"	
	Descending colon—contents	2 i c 24 i p 6 i.n	Rh 2-85	12 13, 15, 16	17-18 (LL part RL + A)	K 18	Typical (olfactory +)	
7	Motor cortex	2 i c (r7) 43 i p	Rh 3-33	12 13, 14	14-17 (part LL Wk RL)	K 17	Typical	
	Descending colon—contents	20 i p 114 i n 6 i p	Rh 2-01	0	12-13 (RL Aph part LL + A)	K 13	Typical (olfactory—0)	

§ *Case 5 Abdominal Sympathetic Plexus*—Histological examination of the nervous system of Rh 3-14 did not satisfy the criteria for a diagnosis of non paralytic poliomyelitis because in none of the six levels of the spinal cord was there any sign of outfall of neurons, interstitial infiltration, or degenerative reaction in the roots There was, however, cuffing of several vessels in the substance of the medulla associated with interstitial glial infiltration in the reticular substance of one side and in the region of one of the vagal nuclei on the other Consequently, a piece of the medulla which had been saved in 50 per cent glycerol in the refrigerator, was passaged to two *rhesus* monkeys One of these developed typical poliomyelitis on the 14th day with complete flaccid paralysis of both legs, and partial paralysis of the arms, it was sacrificed on the 15th day when the temperature dropped from 104 8–101° Microscopically there was extensive neuronal necrosis, neuronophagia, and cuffing in the spinal cord and medulla Passage of material from this monkey again produced paralytic poliomyelitis in another monkey, and the virus was not pathogenic for mice

with marked proliferation of mononuclear cells and epitheloid cell elements, the latter fusing to form small giant cells Examination of Meissner s and Auerbach s plexuses in the ileum suggested that a number of these nerve cell collections especially those of the myenteric plexus which were more readily discerned, had undergone necrosis

Case 3 (Indiana) —Hoo , 16 year old white boy Onset Aug 13, 1940, with fever sore throat headache Aug , 14 same Aug 15 up and about during the day and attended fair at night fever, stiff neck and headache Aug 16 same and vomiting Aug 17, dysphagia, *on admission to hospital, temperature 104 , stiff back* dysphagia, and pleocytosis of 27 cells, became very restless, vomited and developed difficulty in respiration Aug 18, fibrillary twitching noted over face and chest died 6 30 a m

Body in refrigerator, necropsy begun 8 hours after death In the gross there was marked enlargement of the cervical, mesenteric, axillary, and inguinal lymph nodes Microscopically one level of the medulla showed extensive neuronophagia and cuffing affecting chiefly the nuclei ambigui and other nuclei of 9th and 10th cranial nerves, the hypoglossal nuclei and the reticular substance, there was also extensive neuronophagia and cuffing in a midcervical section of the spinal cord, focal neuronophagia and cuffing in a midthoracic section but no changes whatever in a midlumbar region A section of the abdominal sympathetic plexus revealed two questionable minute foci of interstitial and perivascular infiltration with mononuclear cells in one of the ganglia

Case 4 (Indiana) —Wen 13 year old white boy Onset July 27, 1940, with sore throat July 22, p m vomited speech difficulty (nasal quality and poor enunciation) and dysphagia July 29 temperature 103° walked into hospital, obviously ill very restless, stiff neck dysphagia dysphonia and very questionable facial and hypoglossal nerve involvement, reflexes present though hypoactive, pleocytosis of 80 cells July 30, 4 a m 103 6°, secretions *accumulating in back of throat, vomited had tonic convulsion* and opisthotonos terminally, died about 6 a m

Body refrigerated until necropsy was begun 8 hours after death Microscopically there was extensive necrosis neuronophagia and cuffing affecting most of one side and part of the other side of a midcervical section of the spinal cord, and focal neuronophagia and cuffing in the midthoracic and midlumbar levels, one section of medulla at level of decussation of pyramids showed only diffuse cuffing

Case 5 (West Virginia) —Bon 16 year old white boy Onset Aug 21, 1940, with headache and stiffness of neck coming on in the evening after he played baseball all day Aug 22, left work and walked into hospital because someone frightened him by saying that he might have 'polio,' temperature 99 4 , neck and back stiff and painful reflexes variable, some hyperactive and others elicited with difficulty cerebrospinal fluid clear, under normal pressure qualitative test for globulin negative and 20 white cells (mostly polymorphonuclear) per c mm Aug 23 marked dysphagia and dyspnea but no paralysis of extremities Aug 24, hoarse speech and crowing respiration with laryngeal airway patent placed in respirator and 350 cc of convalescent blood given intravenously Aug 25 dysarthria dysphagia and dyspnea increased in severity died 3 30 a.m

Body at room temperature necropsy begun 8 hours after death Microscopically there was extensive neuronal destruction cellular infiltration and cuffing affecting

chiefly both nuclei ambigui and the reticular substance in one section of the medulla One section through the midcervical spinal cord showed extensive destruction of neurons, neuronophagia and cuffing with few nerve cells left intact, the midthoracic and midlumbar sections revealed many foci of neuronophagia and heavy cuffing but most nerve cells were preserved

Case 6 (*West Virginia*) —Pers , 3 year old white girl Onset Aug 7, 1940, with sore throat and "some fever " Aug 10, complete left facial paralysis, some stiffness of neck and back, and 165 white cells per c mm of cerebrospinal fluid Aug 11, dysphagia, generalized weakness of extremities, and somewhat stuporous Aug 13, comatose in a m , temperature 106°, and 90 cells per c mm of cerebrospinal fluid, died 4 30 p m

Necropsy begun 2½ hours after death Poliomyelitis lesions in medulla and spinal cord Since the first inoculation of the spinal cord and medulla into *rhesus* monkeys yielded negative results, it was decided to inoculate the remaining tissues into as many *cynomolgus* monkeys as could be obtained at the time

Case 7 (*Ohio*) —Roo , 15 year old white girl Onset Aug 21, 1940, with headache and backache Aug 22, anorexia, vomiting, constipation, stiff neck and back Aug 23, paralysis of the upper and lower extremities, and difficulty in breathing requiring artificial respiration, on admission to the hospital some movement was found in the left arm and leg but the respiratory excursion was nil, temperature 102 5–104° Aug 24, temperature 101 8–104°, complete paralysis of both upper and lower extremities, weakness of muscles supplied by the left 5th and 12th cranial nerves Aug 25 and 26, temperature 102 6–102 8°, pulse 120 to 145, periodic cyanosis, dysphagia, incontinent of urine and feces Aug 27, died 1 45 a m

Body refrigerated until necropsy was begun 10 hours after death Microscopically all levels of the spinal cord showed the most extensive destruction of neurons, neuronophagia, interstitial infiltration, cuffing, and meningeal infiltration, in the lumbar and thoracic levels an occasional nerve cell was still present, while in the cervical region the destruction appeared to be complete The single level of the medulla examined exhibited heavy cuffing and focal neuronophagia which was especially concentrated on one side in an area corresponding to the nucleus solitarius

This case was remarkable for the fact that no virus was demonstrated in the spinal cord despite the fact that 3 monkeys—2 *rhesus* and 1 *cynomolgus*—were inoculated with large amounts of the tissue The isolation of virus from the motor cortex and the colon contents suggests that one is not dealing with a special strain of virus of low pathogenicity for monkeys but rather that there may perhaps be relatively rapid destruction of the virus where the inflammatory and phagocytic reaction is especially marked

Case 8 (*Indiana*) —Hun , 6 year old white boy Aug 26, 1940, headache, given castor oil for constipation Aug 27, 28, 29, apparently well, soaked in rain on Aug 28 Aug 30, headache, examination by local physician revealed only "bowel trouble " Sept 1, vomiting and "spitting of foamy material," dysphagia, and stiff and painful neck Sept 2, admitted to hospital, unable to swallow or talk well, neck rigid and Kernig sign positive, all reflexes present, 14 white cells per c mm of cerebrospinal fluid, temperature 104 6–105 6° Sept 3, became cyanotic, in respirator, died 10 45 a m

Body refrigerated until necropsy which was begun 7 hours after death The only

remarkable findings in the gross were that the tonsils were necrotic and cheesy" and the cervical lymph nodes were more markedly enlarged than any other group Microscopically, there was neuronophagia of many anterior horn cells and cuffing in the mid lumbar section, focal neuronophagia in the midthoracic, and neuronophagia of most anterior horn cells on one side and of many on the other side, as well as cuffing and meningeal infiltration in the midcervical section of the spinal cord The single section of the medulla revealed heavy cuffing and focal neuronophagia which was especially concentrated in the dorsal motor nucleus of the vagus

Search for Virus—Despite the unquestionable typical lesions, the short duration of the paralysis, preservation of the tissues in the frozen state immediately after their removal from the body, inoculation of the spinal cord into 3 monkeys (1 *cynomolgus*, and 2 *rhesus*) of the medulla and pons, and both olfactory bulbs into one monkey each, did not yield any evidence of the presence of virus Mice inoculated with the human medulla and pons suspension remained well While all the other tissues listed in Table I were obtained in this case they were not tested The descending colon was free of contents

Case 9 (Indiana)—Rip , 21 year old white female nurse who became ill 3 days after she started to take care of a patient with poliomyelitis Onset Aug 8 1940 fainting attack sore throat Aug 9 ached all over dizzy, sore throat Aug 10 nausea, 'rhinitis" Aug 11, difficulty in talking, nausea neckache, and pain between the shoulders Aug 12 rigidity of neck and back, local physician found paralysis of the throat and 130 white cells (70 per cent polymorphonuclear) per c mm of cerebrospinal fluid Aug 13, weakness of right shoulder and irregular shallow respiration 360 cc of convalescent serum administered Aug 14, admitted to hospital and placed in respirator, unable to swallow, talked poorly, cyanotic On Aug 18 left arm weak, Aug 20 became irrational Aug 22, 'the heart beat became rapid, ' respirations short rapid and not in rhythm with the respirator Aug 23, cyanosis and collapse with pulse slowed to 50, died 9 40 a m Fever present throughout and varied from 100–103 6°

Body refrigerated until the necropsy was begun 5½ hours after death Microscopically, one section of the medulla revealed a symmetrical necrotic lesion (older than any of the others) at the site of both nuclei ambigui in addition to heavy cuffing and more recent foci of neuronophagia In the midcervical section of the spinal cord there was practically complete destruction of the anterior horn cells of one side with less involvement on the other side, the anterior horns were also markedly affected in the thoracic section, and while distinct neuronal lesions were present in the lumbar cord more cells were spared here than in the other levels

Search for Virus—This case was especially included in this series to determine whether or not the spread of virus is more diffuse when the disease had lasted longer However since no virus was demonstrated in the spinal cord which was inoculated into 3 monkeys (1 *cynomolgus* and 2 *rhesus*) in the medulla and pons, and the olfactory bulbs, the remaining tissues were not investigated 10 young mice, inoculated with the medulla and pons suspension all remained well The colon contents which weighed only 1 4 gm were suspended in 20 cc of distilled H O, 15 cc of the etherized centrifuged preparation was injected intraabdominally and 1 cc. of the resuspended untreated sediment was instilled intranasally on two successive days into *Rh 3 50* The monkey remained well and histological examination revealed no poliomyelitis lesions

Case 10 (Illinois) —The necropsy on this case was performed by Dr Victor Levine of Chicago who sent us both olfactory bulbs and part of the cervical spinal cord in 50 per cent glycerol in separate bottles as well as the history of the patient and the histological sections

The patient was a 22 year old white woman Onset Sept 10, 1940, with headache, pain in the back and upper thighs, general malaise, anorexia, and occasional nausea and vomiting Paralysis developed Sept 11, and on admission to the hospital on Sept 13 she showed almost complete paralysis of both legs and of the abdominal muscles, poor excursion of the lower ribs, marked weakness of both arms, head drop, and stiff neck, temperature 101 4°, and the cerebrospinal fluid contained 250 white cells per c mm On Sept 13 and 14 she received 380 cc of convalescent serum Respiratory difficulty, cyanosis, and tremors of the arms appeared early Sept 15 and she was placed in a respirator Paralysis finally involved the diaphragm, intercostals, upper extremities, and muscles of deglutition, and the patient died on Sept 16 The necropsy was performed 2½ hours after death Microscopically there was complete destruction of all nerve cells in the lumbar and thoracic sections and of practically all nerve cells in the cervical sections of the spinal cord, with extensive cuffing, interstitial infiltration and neuronophagia Similar lesions, though less extensive, were present in the medulla

Tissues Tested for Virus

Spinal Cord —2 cc i c and 11 cc i p into Rh 2-37 Temperature below 103 5° for first 5 days, 105 5° with excitement and weakness of left hand 6th day, 103 8° with complete paralysis of left upper extremity on 7th day, 101 7° and monkey almost prostrate on 8th day when it was sacrificed Typical severe poliomyelitis lesions

Olfactory Bulbs —Both bulbs ground without an abrasive, suspended in 2 cc distilled water, and injected i c into Rh 2-03 Remained entirely well, sacrificed 35th day, and no poliomyelitis lesions were found

Case 11 (Indiana) —Dau , 2 year and 10 months old white boy Onset Aug 9, 1940, with vomiting and "fever" Anorexia, restlessness and "fever" persisted, paralysis appeared Aug 13 and on admission to the hospital he exhibited left facial paralysis, nystagmus of the right eye, "gurgling in throat," rigidity of neck and back, and convulsive movements, motions and reflexes present in both arms and legs, 28 white cells per c mm of cerebrospinal fluid, temperature 104–106 2° Died Aug 14

Body refrigerated but necropsy not done until 24 hours after death Only few tissues obtained Microscopically there was extensive necrosis of nerve cells, neuronophagia, and cuffing in the medulla, while in the two available sections of the spinal cord, *i e* lower cervical and thoracic, there was chiefly cuffing and almost all cells were preserved

Tissues Tested for Virus

Spinal Cord and Medulla —2 cc i c and 15 cc i p into Rh 1-25 Remained well, sacrificed 36th day, and no poliomyelitis lesions found 10 young mice inoculated with human material remained well

Olfactory Bulbs —Both bulbs ground without abrasive suspended in 1 5 cc physiologic salt solution and injected i c into Rh 1-24 Remained well, sacrificed 36th day, and no poliomyelitis lesions found

Contents of Descending Colon —This material was frozen for 4 months before it was

tested 20 per cent suspension was used and Rh 3-49 received 21 cc i p the first day followed by another i p injection of 20 cc in 24 hours and daily instillations of 1 cc. of untreated suspension into each nostril for 10 days No fever, partial paralysis of both lower extremities 13th day, and complete paralysis of left lower and partial paralysis of all the other extremities 14th day, when the monkey was sacrificed Typical severe poliomyelitis lesions were found in the spinal cord and medulla and their presence in one olfactory bulb and on one side of the anterior perforated substance suggests that the virus invaded by the olfactory pathway

Summary and Analysis of Data

Table III gives some of the pertinent data on the nine complete and two partial poliomyelitis necropsies and shows that while the presence of virus

TABLE III

Human Poliomyelitis Necropsies

Search for Virus in Olfactory Bulbs

Patient	Age	Time since onset of		Primary paralysis	Tests for virus in	
		First symptoms	Paralysis		Olfactory bulbs	Other nervous tissues
	yrs	*days*	*days*			
1 Art.	5	2½	½	U L Fac (terminal bulbar)	0	+
2 Wil.	8	3	1	Bulbar	0	+
3 Hoo	16	5	1		0	+
4 Wen.	13	3	2		—	+
5 Bon	16	4	2		0	+
6 Per	3	6	3		0	+
7 Roo	15	6	4	U L Intercost (terminal bulbar)	0	+
8 Hun	6	4 (8?)	2	Bulbar	*0*	*0*
9 Rip	21	15	12		0	0
10 PM 56 (partial)	22	6	5	U L Intercost (terminal bulbar)	0	+
11 Dau (partial)	21¶	5	1	Bulbar	0	0 (+)

U = upper extremities L = lower extremities Fac = facial

* Virus present in contents of descending colon

was in no instance demonstrated in the olfactory bulbs, it was found elsewhere in the body in all but two cases It is noteworthy and significant that three of the cases which contained demonstrable virus in the nervous system but not in the olfactory bulbs, began with paralysis of the extremities and were not primarily of the bulbar type

The results of tests for virus in seven cases of poliomyelitis in which all the selected tissues were studied are presented in Table IV It is apparent that partly due to the difficulty inherent in the transmission of human virus to monkeys and perhaps also because of the longer duration of the illness in some instances, the virus was not consistently demonstrated in any one tissue However, when the tests on all the cases are pooled a distinct pattern of virus distribution emerges in which certain groups of tissues

yield positive results with considerable regularity while others are consistently negative Thus, in the central nervous system, the olfactory bulbs, the anterior perforated substance with the adjacent corpus striatum, and

TABLE IV

Distribution of Virus in Human Poliomyelitis

Tissues tested	Case No , type and duration of illness						
	1 Spino-bulbar S 2 5 d * Par 0 5 d	2 Bulbar S 3 d Par 1 d	3 Bulbar S 5 d Par 1 d	4 Bulbar S 3 d Par 2 d	5 Bulbar S 4 d Par 2 d	6 Bulbar S 6 d Par 3 d	7 Spino bulbar S 6 d Par 4 d
1 Olfactory bulbs	0	0	0	—	0	0	0
2 Ant perf substance, etc	0	0	0	0	0	0	0
3 Ant. front. + occipit cortex	0	0	0	0	0	0	0
4 Motor cortex	P	P	0	NP	0	0	P
5 Diencephalon	P	P	P	0	0	P^c (+)	0
6 Mesencephalon	0	P	P	NP	0	P	0
7 Medulla (+ pons)	P	NP	P	NP	0	0	0
8 Spinal cord	P (+)	P (+)	P (+)	NP	0 P, P^c	0 NP P^c	0 0, 0^c
9 Sup cerv sympathetic ganglia	0	0	—	0	0	0^c	0
10 Abdominal "	0	0	0	0	NP (+)	0^c	0
11 Suprarenals	0	0	0	0	0	0^c	0^c
12 Salivary glands	0	0	0 (10 d)**	0	0	0^c	0^c
13 Cervical lymph nodes	0	0	0	0	0	0^c	0^c
14 Mesenteric "	0	0	0	0	0	0^c	—
15 Axill + inguin lymph nodes	NP	0	0	0	0	0^c	0^c
16 Lungs + liver + spleen + kidneys	NP (+)	0	0	0	0	0^c	0^c
17 Nasal mucosa	0	0	0	0 (15 d)	0	0^c	0
18 Pharyng mucosa ± tonsils	NP	P (+)‡	0	0	NP	P^c	0
19 Ileum—washed wall	P	0	0, P (+)	0, 0	0	P^c (+)	0
20 —contents	P (+)	P	0	0	0	0	0
21 Desc colon—washed wall	0	0	0	0	0	P^c	0
22 ' ' —contents	Empty	P	P (+)	P (+)	P	P	P

P, paralytic poliomyelitis in inoculated monkey
NP, non-paralytic " " "
0, no evidence of poliomyelitis
c, indicates that a *cynomolgus* monkey was used for the test
(+), passage positive
* S 2 5 d , total duration of illness was 2 5 days
Par 1 d , paralysis 1 day
** (10 d), monkey died on 10th day
‡ No tonsils in this case

the anterior frontal and occipital portions of the neopallial cortex were consistently negative, while the motor cortex, diencephalon, mesencephalon, medulla and pons, and spinal cord were predominantly positive This distribution of virus in the central nervous system is in good agreement not only with the known distribution of neuronal lesions but also with a progression of virus along specific pathways and insulated tracts Next to

these areas in the central nervous system, the virus was predominantly situated in the alimentary tract The pharyngeal mucosa alone or together with the tonsils was positive in four of the seven cases, and it is our belief that the many early positive findings with human tonsils are probably indicative of the presence of virus in the attached pharyngeal mucosa rather than in the tonsils themselves This belief is based not only on the presence of the virus in the pharyngeal mucosa in the absence of tonsils, but also on the consistently negative results with the cervical lymph nodes in this series When one recalls the fact that only a small portion of the entire small intestine was being tested, it is indeed remarkable that the washed wall of the ileum was positive in three cases and the contents in two Very significant also is the finding that the contents of the descending colon contained virus in each one of the six cases in which contents were available, while the washed wall was positive only once The possibility that the presence of virus in the washed intestinal wall might be due to incomplete washing away of the contents was, of course, considered and found to be especially remote as regards the wall of the ileum since the washed wall was positive on two occasions when the total contents of the entire segment were negative In the descending colon, however, the reverse was true and the almost regular presence of virus in the contents suggests that virus originating elsewhere in the alimentary tract is concentrated in the colon

The regularity with which the virus was demonstrated in the contents of the descending colon is not readily accounted for by the methods used, since we did not obtain the same results with the stools of living patients It would appear either that patients in whom the disease terminates fatally have more virus in their intestinal tract, or, and it seems rather unlikely, that something happens to the virus between the descending colon and the rectum

In the next group of tissues one finds that the nasal mucosa, the salivary glands, the superior cervical sympathetic ganglia, the suprarenals, and the cervical and mesenteric lymph nodes were consistently negative whether tested in *rhesus* or *cynomolgus* monkeys This finding suggests a number of points which are of significance in understanding the nature of human poliomyelitis (*a*) that the previously reported finding of virus in the nasal mucosa in a single case of human poliomyelitis represents a result that is either rare and fortuitous or due to contamination, (*b*) that the centrifugal spread of virus, which is so common in rabies, does not appear to occur in human poliomyelitis, (*c*) the negative results with the salivary glands suggest that the virus is not likely to be eliminated by way of the saliva (*d*) the negative results with the superior cervical sympathetic ganglia are an

indication not only of the absence of any appreciable centrifugal spread but also that the virus did not spread centripetally along this sympathetic pathway, (*e*) that virus which is demonstrable in the wall of the pharynx or ileum is probably there because that is its portal of entry and not because it has become established there in a centrifugal spread of the virus, for if the latter were true it should also have been present in the suprarenals, salivary glands, etc , (*f*) that virus which is present in the contents and wall of the alimentary tract is not readily absorbed or is at least not demonstrable in the lymph nodes draining those areas despite the fact that they exhibit distinct pathologic changes, these results with the lymph nodes are in agreement with those of numerous tests by Flexner (3), and the more recent report of Kling, Olin, and Gard (4) in which the virus was presumably demonstrated on rare occasions in the mesenteric (4 of 33 specimens) and cervical (1 of 25 specimens) lymph nodes may perhaps be accounted for partly on the basis that their criteria for experimental poliomyelitis are in some cases not acceptable (5) and partly that the virus may actually get get into these lymph nodes on rare occasions It is of interest in this respect that in mice which normally carry the virus of mouse polioencephalitis in their intestinal tract, Olitsky (12) found that the infective agent although present on occasion is not readily demonstrable in the mesenteric lymph nodes

The presence of virus in the pool of axillary and inguinal lymph nodes and in the pool of the lungs, liver, spleen, and kidneys in the first case in which the patient died after an illness of only $2\frac{1}{2}$ days, at first suggested that the virus may perhaps be more widely distributed in the early stages of the disease Until further data become available, however, the following alternative interpretation appeals to us more (*a*) that the pool of lymph nodes was positive not because of the axillary nodes but because of the inguinals which drain the perianal region where virus from the intestinal contents might enter through a fissure, and (*b*) that the lungs containing aspirated material from the alimentary tract probably contributed the virus to the pool of the viscera

Although the abdominal sympathetic plexus was tested in all seven cases, five of which were of the bulbar type and two spino-bulbar, evidence of its presence was obtained in only one instance and that in a bulbar case While the monkey inoculated with the human material developed neither paralysis nor lesions in the spinal cord and exhibited only interstitial infiltration and perivascular cuffing in the medulla, the production of the typical paralytic disease on passage can leave little doubt of the presence of virus in the abdominal sympathetic plexus of this case It will be necessary to obtain

additional data with the abdominal sympathetic ganglia (dissected with especial care to include its superior mesenteric component—and we cannot be certain that we always had this component in the present study) and especially from cases with ascending paralysis, which rarely terminate fatally, before much can be said about the significance of this finding How ever, it is hardly necessary to stress that the presence of virus in the ab dominal sympathetic plexus, under conditions pointing against generalized centrifugal spread, is in good agreement with progression of the virus from the lower portion of the alimentary tract along the sympathetic pathway Should further investigations confirm the suggestion established by the present studies that human poliomyelitis is a disease in which the alimentary tract is primarily attacked with secondary involvement of the central nervous system, it is clear that, in the many instances in which only the lower extremities are affected, progression of the virus along the sympathetic fibers of the intestines through the abdominal sympathetic plexus is defi nitely to be expected It is noteworthy, however, that even in the strictly bulbar type of the disease, the virus may be present not only in the walls of the pharynx but also in the ileum and colon

DISCUSSION

Ever since human poliomyelitis was shown to be an infectious disease, various concepts of its essential nature have been entertained Thus, it has been variously regarded (*a*) as a disease in which the virus multiplies in the upper respiratory tract and from there both invades the nervous system and escapes to infect others, (*b*) as a disease in which peripheral multiplication of the virus and the site from which the nervous system is invaded is limited to the olfactory portion of the nasal mucosa, (*c*) that the virus may perhaps enter by way of the skin either by means of an insect bite or direct contamination, and (*d*) as primarily a gastro enteric infection partly because of its occurrence chiefly in the late summer and early autumn and the finding of virus in the stools, and partly because of the results of certain animal experiments The data upon which the various hypotheses were based originated only in small part from direct studies on the human disease, and were derived mostly from observations on the behavior of the virus chiefly in *rhesus* monkeys and from certain epidemiological observa tions which could be interpreted in a number of different ways

The upper respiratory tract hypothesis was based chiefly on the demon stration that virus could occasionally be isolated from nasopharyngeal washings (note the pharyngeal component) that it was present in the human tonsils (there was always attached pharyngeal mucosa), that it was pre

sumably isolated in a single instance from the human nasal mucosa, and on the fact that next to intraneural injection, infection of the *rhesus* monkey was most readily accomplished by nasal instillation of the virus When it was found in recent years that virus instilled intranasally in *rhesus* monkeys could invade the nervous system only by the olfactory pathway, the belief became almost general that the same was true of human poliomyelitis It was then shown that distinct lesions were regularly present in the olfactory bulbs of monkeys when the nervous system was invaded by the olfactory pathway but not when the virus entered in any other way (9) These lesions which were thus indicators of the olfactory portal of entry were not found, however, in the human olfactory bulbs despite the fact that thousands of sections were studied (10) The skin as the site of primary attack was suggested by the presumably greater infectivity by the intracutaneous route of some freshly isolated strains (13), and by the occurrence of poliomyelitis following subcutaneous injection of certain vaccines in human beings (14) This hypothesis was obviously incomplete in explaining poliomyelitis as an epidemic disease which is presumably limited to man The gastro-enteric hypothesis has been through many vicissitudes In 1912, Kling, Wernstedt, and Pettersson (15) obtained the virus (proved by positive passage in at least one case) from the washings of the small intestine in three of six fatal cases of human poliomyelitis, but they also reported that in the same series of cases the virus was present in the combined washings from the mouth and nose, as well as in the washings of the trachea This did not elucidate the origin of the virus and its presence in the washings of the small intestine was usually interpreted as representing virus that had been swallowed However, the clues contained in the work of Kling, Wernstedt, and Pettersson were not pursued and remained dormant for a quarter of a century chiefly because in their subsequent work on intestinal contents and nasopharyngeal washings from patients and contacts it became apparent that they often employed criteria for the presence of virus which were later found not to be acceptable The whole work was set aside without attempting to extract the undeniably significant and valid results In the past few years, however, with the development of more adequate methods, incontrovertible evidence, has been brought forth for the presence of virus in the stools of patients with paralytic or non-paralytic poliomyelitis and in contacts as well (16) This important finding, however, threw little light on the essential nature of the human disease since there was as yet no indication that the virus in the stools did not have its origin in swallowed secretions Experimental work on monkeys has until recently contributed little or nothing in support of the gastro-enteric hypothesis The work on

rhesus monkeys in which poliomyelitis was produced by injecting large amounts of virus into the gut (17) was not accepted partly because it was not readily reproduced (3, 18) and partly because it did not approximate natural conditions The results of earlier feeding experiments on *cynomolgus* monkeys were irregular and also inconclusive because there was no evidence that infection under those conditions had not occurred by the olfactory pathway The more recent experiments of Burnet, Jackson, and Robertson (1) indicated that *cynomolgus* monkeys could probably be infected by the oral route without involving the olfactory pathway, and Howe and Bodian (2) established that chimpanzees, whose olfactory tracts had been severed, could develop poliomyelitis after being fed human stools containing the virus

The results of the present investigation on the distribution of virus in the tissues of fatal cases of human poliomyelitis permit a revaluation of the various hypotheses in the light of the following findings in the human disease

1 The absence of demonstrable virus in the olfactory bulbs and anterior perforated substance indicates that the olfactory pathway need not be affected in human beings

2 The absence of infective virus in the nasal mucosa suggests that it is not the site of virus multiplication and dissemination

3 The absence of virus in the salivary glands indicates that the saliva is not a likely means for its elimination

4 The positive results with the tonsils and pharyngeal mucosa, are probably due to the pharyngeal tissue rather than the tonsils

5 Next to the central nervous system the virus is distributed predominantly in the alimentary tract and is present not only in the contents but also in the washed walls of various parts of the tract including the pharynx, ileum, and occasionally the colon

6 Infection of the walls of the alimentary tract appears to be the result neither of generalized dissemination of the virus nor of secondary centrifugal spread, but rather that of primary localization or portal of entry

7 The distribution of virus in the central nervous system is limited to certain areas and is not as indiscriminately disseminated as viruses (*e g*, equine encephalomyelitis) which can invade through the blood vessels or those (*e g*, rabies), which having entered by a specific nervous pathway, are capable of extensive centrifugal spread

8 In the absence of evidence of any appreciable centrifugal spread to peripheral collections of nerve cells, the demonstration of virus in the abdominal sympathetic ganglia of one case is significant in suggesting one of the possible routes of virus progression in certain instances

The pattern of virus distribution in human poliomyelitis, as it emerges from the present study, thus points to almost the entire alimentary tract as the primary site of attack by the virus, and contains no support for the concepts involving either the olfactory pathway or the respiratory tract This pattern of virus distribution also militates against the cutaneous route although it is conceivable that occasional infection by direct contamination of the broken skin may be possible It is of interest to note how much similarity there appears to be between this picture of human poliomyelitis and that of Theiler's spontaneous mouse encephalomyelitis or poliomyelitis, in which Olitsky (19) first demonstrated that the virus is carried in the intestinal tract of many normal mice, although only 1 of 2000 to 5000 develops paralysis The subsequent demonstration by Theiler and Gard (20) and by Olitsky (12) that the infectious agent is also present in the washed intestinal wall and that this is the most probable origin of the virus in the intestinal contents, brings out a most remarkable analogy between the two diseases

CONCLUSIONS

1 Studies on a large number of tissues obtained from fatal cases of human poliomyelitis have revealed that the virus is distributed predominantly in two systems (*a*) certain regions of the nervous system, and (*b*) the alimentary tract

2 Poliomyelitis virus was demonstrated in the walls of the pharynx, ileum, and only once in those of the descending colon, while the contents of the descending colon regularly contained the virus

3 The presence of virus in the walls of the alimentary tract appears to be the result neither of generalized dissemination of the virus nor of secondary centrifugal spread, but rather that of primary localization or portal of entry

4 In the absence of evidence of any demonstrable centrifugal spread to peripheral collections of nerve cells (*e g*, in the superior cervical sympathetic ganglia, suprarenals, salivary glands), the presence of virus in the abdominal sympathetic plexus of one case may be indicative of at least one pathway of centripetal virus progression

5 The absence of demonstrable virus in the nasal mucosa, olfactory bulbs, and anterior perforated substance suggests that neither the upper respiratory tract nor the olfactory pathway were affected in the cases of human poliomyelitis studied in the present investigation

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